

membranes. HeLa cells transiently transfected with cytoplasmic S-F<sub>v</sub>2-Casp3-E (C), plasma membrane-localized M-F<sub>v</sub>2-Casp3-E (D), mitochondria-localized Mas70<sub>34</sub>-F<sub>v</sub>2-Casp3-E (E), or nuclear N2-F<sub>v</sub>2-Casp3-E (F) were fixed, stained with anti-HA antibodies and examined by confocal microscopy. Alternatively, control proteins were localized, including S-F<sub>v</sub>2-E (G), M-F<sub>v</sub>2-E (H), Bcl-x<sub>L</sub>-E (I), or Gal4-VP16-E (J). In each case, cells shown are representative of several transfected cells.

Figure 27. Nuclear-targeted caspase-1, -3, and -8 trigger apoptosis. (A-B) Transient transfection assay was performed as above. (A and B) Cells received 2 μg SRα-SEAP plus 2 μg nuclear targeted caspases, including N2-F<sub>v</sub>1-F<sub>vis</sub>1-Casp1 (Δ), N2-F<sub>v</sub>1-F<sub>vis</sub>1-Casp3 (▲), N2-F<sub>v</sub>1-F<sub>vis</sub>1-Casp8 (◻), or control construct N2-F<sub>v</sub>1-F<sub>vis</sub>1 (◻). (C) Nuclear targeted caspase-3 functions efficiently. Cells received SRα-SEAP plus 4 μg (◻) or 1 μg (Δ) S-F<sub>v</sub>2-Casp3, 4 μg (◻) or 1 μg (▲) N2-F<sub>v</sub>2-Casp, or 1 μg S-F<sub>v</sub>1-F<sub>vis</sub>1 (○). (D) Nuclear targeted FADD<sub>125</sub> has reduced activity. Cells received reporter plasmid plus 2 μg S-F<sub>v</sub>1-F<sub>vis</sub>1-FADD<sub>125</sub> (◻), N2-F<sub>v</sub>1-F<sub>vis</sub>1-FADD<sub>125</sub> (◻), or N2-F<sub>v</sub>1-F<sub>vis</sub>1 (Δ).

Figure 28. A) miniprep gel of pAdTrack-CMV (9220bp); B) miniprep gel of pAdTrack-CMV, pSH1/S-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst and pSH1/S-F<sub>vis</sub>1-Yama-E;

Figure 29. A) Representation of pAdTrack-CMV; B) and C) Gel of miniprep check of pAdTrack-CMV-S-F<sub>vis</sub>1-Yama-E.

Figure 30. A) Representation of pAdTrack-CMV; B), C) and D) Gel of miniprep check of pAdTrack-CMV-S-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst.

Figure 31. A) Gel of miniprep check of pSH1/S-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst and B) Representation of pSH1/S-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst;

Figure 32. Representation of pSH1/S-F<sub>vis</sub>1-Yama-E;

Figure 33. Outline of the Generation of ADV-GFP-CMV-Yama-E and ADV-GFP-CMV-E-ICE;

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- Figure 34. Schematic diagram showing general protocol for generation of ADV-GFP-CMV-Yama-E and ADV-GFP-CMV-E-ICE;
- Figure 35. A) Representation of pADEasy-1 and B) Gel of miniprep check of pADEasy-1;
- 5 Figure 36. A) Gel of miniprep check of pADEasy-1-Track-CMV-E-ICE; B) and C) Gels of miniprep checks of pAdEasy-1-Track-CMV-Yama-E;
- Figure 37. A) miniprep gel check of colony #2 of pShuttle-CMV-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst; B) miniprep gel check of pShuttle-CMV-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst from multiple colonies of transformed E.coli BJ5183 competent cells;
- 10 Figure 38. Schematic representation of protocol for ADV-CMV-E-ICE;
- Figure 39. A) Representation of pShuttle-CMV and B) miniprep gel;
- Figure 40. Results of Luciferase assay of pShuttle-CMV-F<sub>v</sub>1-F<sub>vis</sub>1-ICE-E;
- Figure 41. Outline of Assay of Effect of Ad-YAMA and Ad-ICE on Different cell types;
- 15 Figure 42. Graph showing effect of Ad-YAMA and Ad-ICE on T-C2G cells;
- Figure 43. Graph showing effect of Ad-YAMA and Ad-ICE on T-C2 cells;
- Figure 44. Graph showing effect of Ad-YAMA and Ad-ICE on JD-2a cells;
- Figure 45. Graph showing effect of Ad-YAMA and Ad-ICE on LNCaP cells;
- Figure 46. Outline and Western Blot showing expression and activation of ICE and YAMA;
- 20 Figure 47. A) untreated JD-2a cell culture; B) control culture incubated with Adv-Fv1-YAMA expressing green fluorescent protein; C) cell culture incubated with virus and maintained in 50nM AP1903;
- Figure 48. Plated PC-3 cells incubated with ADV-FKBP/ICE and treated (+) or untreated with AP1903 at increasing MOI;
- 25 Figure 49. Plated JD-2a BPH cells incubated with ADV-FKBP/ICE and treated (+) or untreated with AP1903 at increasing MOI;
- Figure 50. Diagram illustrating protocol for treatment of s.c. prostate adenocarcinoma in situ with CID inducible caspases;

## EXAMPLE 17

### Construction of an adenovirus expressing a chemically inducible apoptosis factor.

5 The E-F<sub>v</sub>2 YAMA-E construct described previously was placed under the control of the CMV promoter and inserted into an adenoviral recombination vector. The adenoviral recombination vector contained a copy of the D1 gene from adenovirus. The CMV promoter - E-F<sub>v</sub>2-YAMA-E construct was inserted such that the F<sub>v</sub>2 construct was flanked on both sides by nucleotide sequences from the E1 gene.

10 The plasmid was transfected into adenovirus infected cells and a recombinant adenovirus expressing the chemically inducible apoptosis factor was isolated and purified by standard methods.

15 Figure 19 A shows a plasmid map of the adenoviral recombination vector used to construct an adenovirus expressing E-F<sub>v</sub>2-YAMA-E under control of the CMV promoter and incorporating the 16S splice junction to improve the efficiency of mRNA processing (Takabe, *et al.* Mol. Cell. Bio. 8:466-472, 1988). Panel B shows the results of a restriction analysis of the plasmid. Figure 20 A shows a plasmid map of the plasmid used to construct a recombinant adenovirus expressing E-F<sub>v</sub>2-YAMA-E under the control of the SR $\alpha$  promoter. Panel B shows the results of a restriction analysis of the plasmid.

## EXAMPLE 18

### Construction of Additional Adenovirus Constructs Containing Conditional Caspase 1 or 3

- 20 I. Construction of pAdTrack-CMV-F<sub>vis</sub>1-Yama-E (Fig. 28)
1. Digest pAdTrack-CMV (Fig. 29A) with Eco RV and Not I. Purify the 9.2 kb vector by agarose electrophoresis and GeneClean (Fig. 28 B).
  2. Digest pSH1/S-F<sub>vis</sub>1-Yama-E (Fig. 32) with Eco RI and Not I. Blunt the Eco RI end. Purify the 1.2 kb fragment by agarose electrophoresis and GeneClean.
  - 25 3. Ligate the above two fragments and transform XL-1 Blue with the ligation. Chose several colonies, do Miniprep and check with Sal I, Not I + Eco RI, Hind III + Eco RI, Hind III + Xho I. See Figs. 29 B and 29 C.

II. Construction of pAdTrack-CMV-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst (See Fig. 28)

1. Digest pAdTrack-CMV with Eco RV and Not I. Purify the 9.2 kb vector by agarose electrophoresis and GeneClean (Fig. 28 B).
2. Digest pSH1/S-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst (Fig. 31 B) with Eco RI and Not I. Blunt the Eco RI end. Purify the 2.2 kb fragment by agarose electrophoresis and GeneClean.
3. Ligate the above two fragments and transform XL-1 Blue with the ligation. Chose several colonies, do Miniprep and check with Sal I, Not I + Eco RI, Hind III + Eco RI, Hind III + Xho I. See Figs. 30 B through 30 D.

III. Generation of ADV-GFP-CMV-Yama-E (Figs. 33 and 34)

1. Linearize 1  $\mu$ g pAdTrack-CMV-F<sub>vis</sub>1-Yama-E with Pme I. Purify it by phenol-chloroform extraction, ethanol precipitation and resuspend in 6  $\mu$ l H<sub>2</sub>O.
2. Mix it with 100  $\mu$ g p AdEasy-1 (Fig. 35) (in 1  $\mu$ l), Co-transform 20  $\mu$ l E. coli BJ5183 competent cells with GenePulser at 2,500 V, 200 Ohms, 25 uFD.
3. Pick up 20 smallest colonies. Do Miniprep and check with Pac I. Candidate clones usually yield a large fragment (near 30 kb), plus a smaller fragment of 3.0 kb or 4.5 kb.
4. Re-transform the correct recombinant plasmids into XL-1 Blue. Midiprep with Qiagen kit.
5. Transfect 293 cell by the recombinant plasmid with FuGene. 4 $\mu$ g DNA/6 $\mu$ l FuGene/well (6 well-plate). Check GFP expression with fluorescent microscope.
6. Harvest the cells when 30% of them are detached. Spin down the cells; use the supernatant for next infection. Repeat infection for several rounds.
7. Collect the cells, repeat freeze/thaw/vortex four times. Purify the virus by CsCl gradient centrifuge.

IV. Generation of ADV-GFP-CMV-E-ICE (Figs. 33 and 34)

1. Linearize 1  $\mu$ g pAdTrack-CMV- E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst with Pme I. Purify it by phenol-chloroform extraction, ethanol precipitation and resuspend in 6  $\mu$ l H<sub>2</sub>O.
2. Mix it with 100  $\mu$ g p AdEasy-1 (Fig. 35 A) (in 1  $\mu$ l), Co-transform 20  $\mu$ l E. coli BJ5183 competent cells with GenePulser at 2,500 V, 200 Ohms, 25 uFD.
3. Pick up 20 smallest colonies. Do miniprep and check with Pac I. Candidate clones usually yield a large fragment (near 30 kb), plus a smaller fragment of 3.0 kb or 4.5 kb.
4. Re-transform the correct recombinant plasmids into XL-1 Blue. Midiprep with Qiagen kit.
5. Transfect 293 cell by the recombinant plasmid with FuGene. 4 $\mu$ g DNA/6 $\mu$ l FuGene/well (6 well plate). Check GFP expression with fluorescent microscope.
6. Harvest the cells when 30% of them are detached. Spin down the cells, use the supernatant for next infection. Repeat infection for several rounds.
7. Collect the cells, repeat freeze/thaw/vortex four times. Purify the virus by CsCl gradient centrifuge.

V. Construction of pShuttle-CMV-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst (Figs. 37-39)

1. Digest pShuttle-CMV (Fig. 39) with Eco RV and Not I. Purify the 7.4 kb vector by agarose electrophoresis and GeneClean.
2. Digest pSH1/S-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst with Eco RI and Not I. Blunt the Eco RI end. Purify the 2.2 kb fragment by agarose electrophoresis and GeneClean. Fig. 38.
3. Ligate the above two fragments and transform XL-1 Blue with the ligation. Chose several colonies, do miniprep and check with Sal I and Eco RI.

VI. Generation of ADV-CMV-E-ICE (Figs. 37-39)

## EXAMPLE 20

### Assay To Determine Effects of Using Different Viruses (Figs. 41-45)

(See Table 4 for a more detailed protocol).

1. Cells were plated in 24 well-plate,  $2 \times 10^4$  to  $4 \times 10^4$  cells/well in 1 ml media (RPMI 1640 for JD-2 $\alpha$ , LNCaP and PC3 cells; DMEM for Tramp, T-C2 and T-C2G cells) with 5% FBS, and incubated until the cell number doubled.
2. Cells were infected with the virus at different MOI and incubated overnight.
3. AP1903, or FK 1012, or AP20187 was added at a final concentration of 50 nM and cultures were incubated 24 hours.
4. Cells were fixed with 1% glutaraldehyde for 15 min.; stained with 0.5% crystal violet for 20 min.; washed with H<sub>2</sub>O for 30 min.; air dried; resolved with 200-500  $\mu$ l/well Soreson's Solution for 5 min.; transferred 60—100  $\mu$ l to each well of 96-well plate and read OD at 570 nm. Results are shown in Figs 42-45.

## EXAMPLE 21

Replication deficient ( $\Delta$  E1) adenoviral vectors expressing green fluorescent protein and conditional Caspase 1 (ICE) or Caspase 3 (YAMA) were engineered. These vectors independently express green fluorescent protein so that infected cells are easily identified by their green color under fluorescent microscopy. These vectors were tested for their ability to induce apoptosis *in vitro* in a SMC line derived from a patient with BPH upon administration of a non-toxic, lipid-permeable, divalent FK506 analog (AP1903).

40,000 JD-2a cells per well were plated in 24-well plates and infected at a multiplicity-of-infection (MOI) of ~25 with Adv-F<sub>v</sub>1-YAMA, an adenoviral vector expressing CID-regulated YAMA. After 24 hours, culture media was changed to control media  $\pm$  50nM AP1903 for an additional 24 hours, and the cells were viewed under fluorescent microscopy. All of the cells incubated with virus and maintained in control media appeared green, and were clearly attached and viable, similar to non-fluorescent

## REFERENCES

1. Moolten, F.L. (1986) *Cancer Res.* **46**, 5276-5281.
2. Culver, K.W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H. & Blaese, R.M. (1992) *Science* **256**, 1550-1552.
- 5 3. Matthews, T. & Boehme, R. (1988) *Rev. Infect. Dis.* **10 Suppl 3**, S490-S494.
4. Ogasawara, J., Suda, T. & Nagata, S. (1995) *J. Exp. Med.* **181**, 485-491.
5. Spencer, D.M., Belshaw, P.J., Chen, L., Ho, S.N., Randazzo, F., Crabtree, G.R. & Schreiber, S.L. (1996) *Current Biology* **6**, 839-847.
6. Liles, W.C., Kiener, P.A., Ledbetter, J.A., Aruffo, A. & Klebanoff, S.J. (1996) *J. Exp. Med.* **184**, 429-440.
- 10 7. Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. & Nagata, S. (1993) *Nature* **364**, 806-809.
8. Belshaw, P.J., Spencer, D.M., Crabtree, G.R. & Schreiber, S.L. (1996) *Chem. Biol.* **3**, 731-738.
- 15 9. Smith, C.A., Farrah, T. & Goodwin, R.G. (1994) *Cell* **76**, 959-962.
10. Yuan, J. (1997) *Curr. Opin. Cell Biol.* **9**, 247-251.
11. Hengartner, M.O. (1997) *Nature* **388**, 714-715.
12. Kroemer, G. (1997) *Nat. Med.* **3**, 614-620.
13. Spencer, D.M., Wandless, T.J., Schreiber, S.L. & Crabtree, G.R. (1993) *Science* **262**, 1019-1024.
- 20 14. Spencer, D.M. (1996) *TIG* **12**, 181-187.
15. Crabtree, G.R. & Schreiber, S.L. (1996) *Trends. Biochem. Sci.* **21**, 418-422.
16. Duan, H., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W. & Dixit, V.M. (1996) *J. Biol Chem* **271**, 1621-1625.
- 25 17. Rosen, M.K., Yang, D., Martin, P.K. & Schreiber, S.L. (1993) *JACS* **115**, 821-822.
18. Spencer, D.M., Graef, I., Austin, D.J., Schreiber, S.L. & Crabtree, G.R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9805-9809.
19. Northrop, J.P., Ullman, K.S. & Crabtree, G.R. (1993) *J. Biol Chem* **268**, 2917-2923.
20. Henkart, P.A. (1996) *Immunity* **4**, 195-201.

21. Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H. & Wallach, D. (1995) *J. Biol. Chem.* **270**, 7795-7798.
22. Irmeler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E. & Tschopp, J. (1997) *Nature* **388**, 190-195.
23. Deveraux, Q.L., Takahashi, R., Salvesen, G.S. & Reed, J.C. (1997) *Nature* **388**, 300-304.
24. Freiberg, R.A., Spencer, D.M., Choate, K.A., Duh, H.J., Schreiber, S.L., Crabtree, G.R. & Khavari, P.A. (1997) *J. Invest. Dermat.* **108**, 215-219.
25. Aggarwal, B.B., Singh, S., LaPushin, R. & Totpal, K. (1995) *FEBS Letters* **364**, 5-8.
26. Freiberg, R.A., Spencer, D.M., Choate, K.A., Peng, P.D., Schreiber, S.L., Crabtree, G.R. & Khavari, P.A. (1996) *J. Biol. Chem.* **271**, 31666-31669.
27. Veis, D.J., Sentman, C.L., Bach, E.A. & Korsmeyer, S.J. (1993) *J. Immunol* **151**, 2546-2554.
28. Pan, G., O'Rourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J. & Dixit, V.M. (1997) *Science* **276**, 111-113.
29. Boldin, M.P., Goncharov, T.M., Goltsev, Y.V. & Wallach, D. (1996) *Cell* **85**, 803-815.
30. Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. & Dixit, V.M. (1996) *Cell* **85**, 817-827.
31. Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S. & Dixit, V.M. (1995) *Cell* **81**, 801-809.
32. Chinnaiyan, A.M., O'Rourke, K., Tewari, M. & Dixit, V.M. (1995) *Cell* **81**, 505-512.
33. Bauer, M.K., Wesselborg, S. & Schulze-Osthoff, K. (1997) *FEBS Lett.* **402**, 256-258.
34. Chinnaiyan, A.M., Chaudhary, D., O'Rourke, K., Koonin, E.V. & Dixit, V.M. (1997) *Nature* **388**, 728-729.
35. Zou, H., Henzel, W.J., Liu, X., Lutschg, A. & Wang, X. (1997) *Cell* **90**, 405-413.
36. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. & Wang, X. (1997) *Cell* **91**, 479-489.



37. Chinnaiyan, A.M., O'Rourke, K., Lane, B.R. & Dixit, V.M. (1997) *Science* **275**, 1122-1126.
38. Wu, D., Wallen, H.D., Inohara, N. & Nunez, G. (1997) *J Biol. Chem.* **272**, 21449-21454.
- 5 39. Boulikas, T. (1997) *Anticancer Research* **17**, 1471-1506.

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What is claimed is:

1. A conditionally lethal molecule comprising a chemical inducer binding domain and an apoptosis inducing factor, wherein said apoptosis inducing factor is an apoptosis signal transducing factor.

2. A conditionally lethal molecule according to claim 1, wherein said  
5 apoptosis inducing factor is an adaptor molecule.

3. A conditionally lethal molecule according to claim 1, wherein said apoptosis inducing factor is a protease.

4. A conditionally lethal molecule according to claim 1, wherein said apoptosis inducing factor is a caspase.

10 5. A nucleic acid molecule encoding the conditionally lethal molecule of any one of claims 1-4.

6. A nucleic acid molecule according to claim 5, further comprising a sequence coding for tissue specific expression operatively linked to a sequence coding for a conditionally lethal molecule.

15 7. A gene therapy vector comprising a nucleic acid sequence coding for the expression of a conditionally lethal molecule according to anyone of claims 1-4.

8. A gene therapy vector according to claim 7, further comprising a sequence coding for a therapeutic gene.

20 9. A gene therapy vector according to claim 7, further comprising a sequence coding for tissue specific expression operatively linked to a sequence coding for a conditionally lethal molecule.

10. A transgenic animal expressing a conditionally lethal molecule according to any one of claims 1-4.

11. A method of making a transgenic animal comprising the step of micro-injecting a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

12. A method of treating a disease comprising the step of administering a vector that encodes a conditionally lethal molecule according to any one of claims 1-4.

13. A method according to claim 12, wherein the disease is a hyperproliferative disease.

14. A method according to claim 13, wherein the hyperproliferative disease is a benign disease.

15. A method according to claim 14, wherein the disease is a malignant disease.

16. A method according to claim 12, wherein the disease is atherosclerosis.

17. A method of causing regression of a tumor comprising transfecting cells of said tumor with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

18. A method according to claim 17 further comprising administering a CID.

19. A method of causing a reduction in tumor size comprising transfecting cells of said tumor with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

5 20. A method according to claim 19 further comprising administering a CID.

21. A method of causing a reduction in PSA levels comprising transfecting cells of a tumor with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

10 22. A method according to claim 21 further comprising administering a CID.

23. A method of affecting the rate of cell proliferation caused by BPH comprising transfecting prostate cells with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

15 24. A method according to claim 23 further comprising administering a CID.

25. A method of inducing apoptosis in a cell comprising transfecting said cell with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

20 26. A method according to claim 26 further comprising administering a CID.

27. A method for determining the biological role of a cell type, comprising transfecting a cell of said cell type with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4 and administering a CID.

## ABSTRACT OF THE DISCLOSURE

The present invention discloses artificial death switches (ADSs) based on chemically induced dimerization of the cysteine proteases, caspase-1 (ICE) and caspase-3 (YAMA). In both cases, aggregation of the target protein is achieved by a non-toxic, lipid-permeable, dimeric FK506 analog that binds to an attached FK506-binding protein (FKBP). The intracellular crosslinking of caspase-1 or caspase-3 is sufficient to trigger rapid apoptosis in a Bcl-x<sub>L</sub>-independent manner, suggesting that these conditional pro-apoptotic molecules can bypass intracellular checkpoint genes, like Bcl-x<sub>L</sub>, that limit apoptosis. Since these chimeric molecules are derived from autologous proteins, they should be non-immunogenic and thus ideal for long-lived gene therapy vectors. These properties should also make chemically-induced apoptosis (CIA) useful for developmental studies, for treating hyperproliferative disorders and for developing animal models to a wide variety of diseases.

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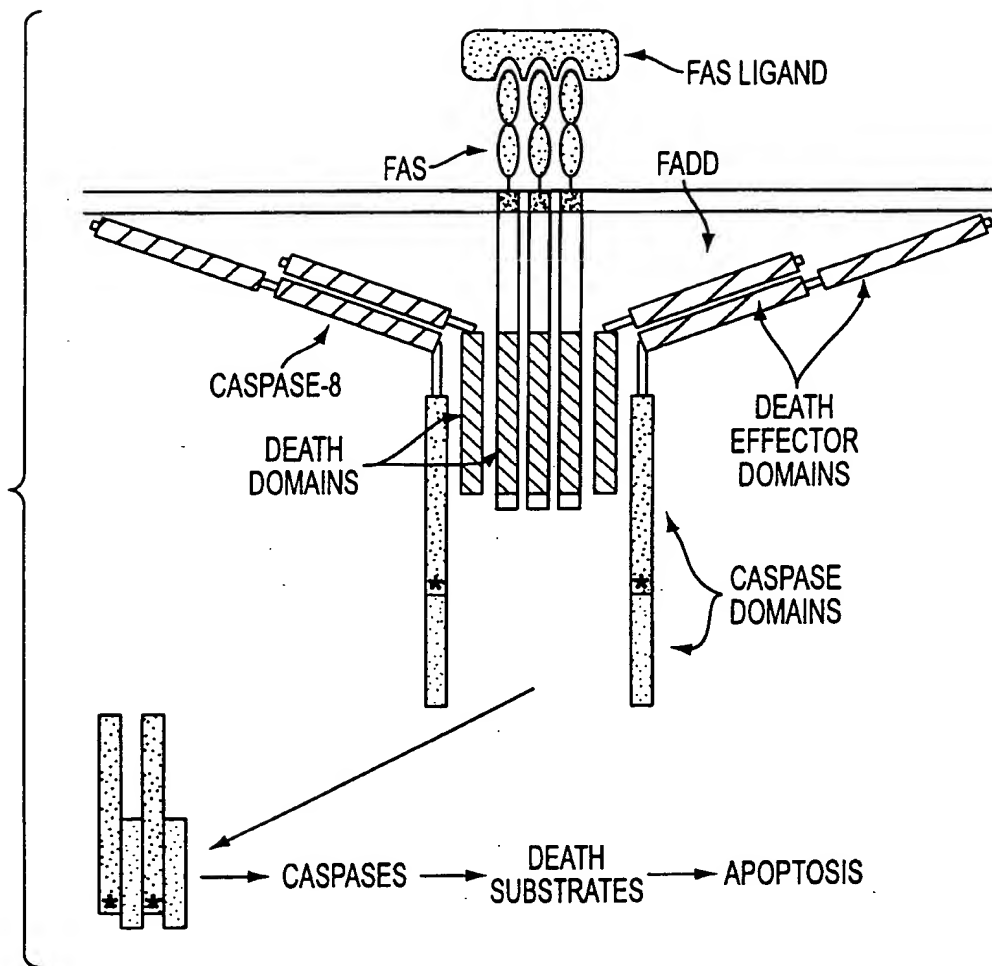


FIG. 1

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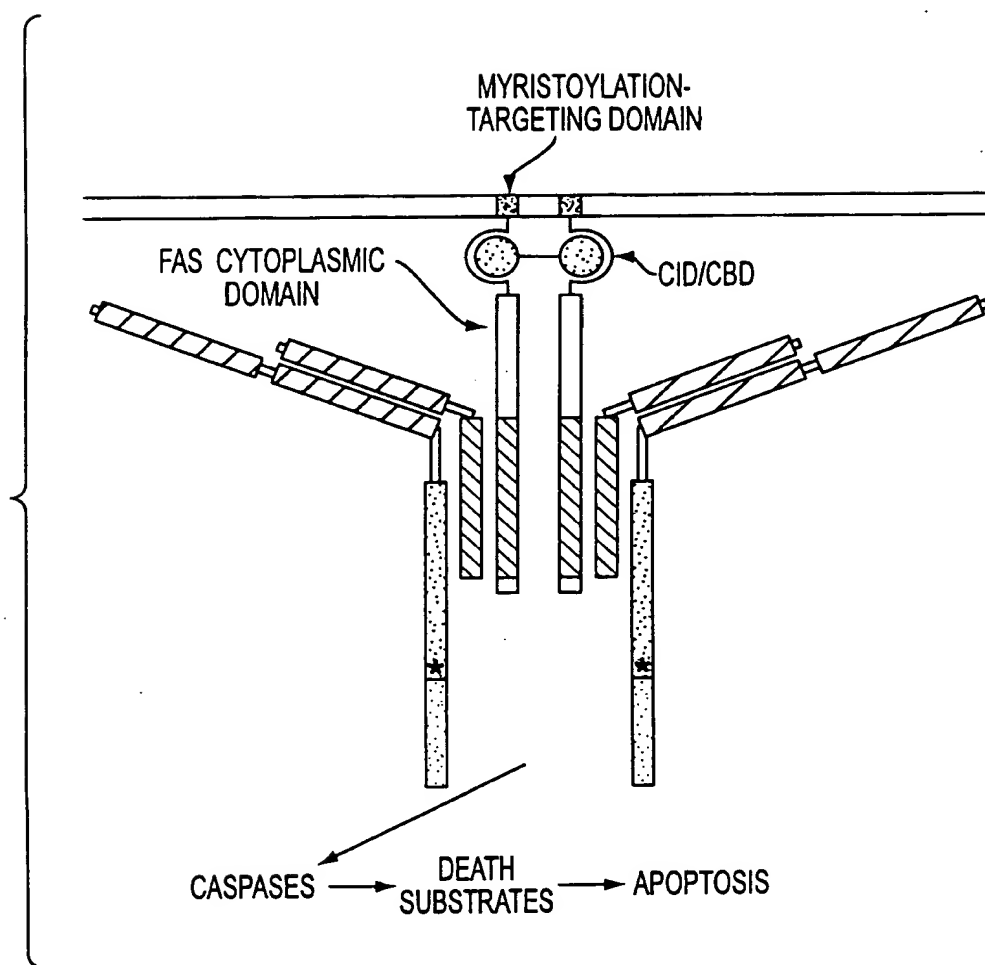


FIG. 2

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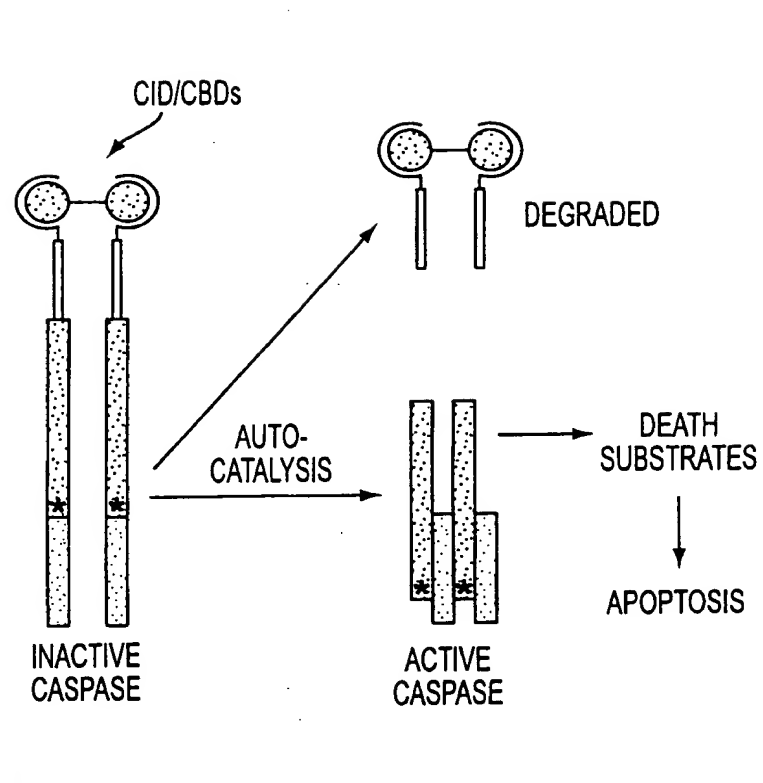


FIG. 3



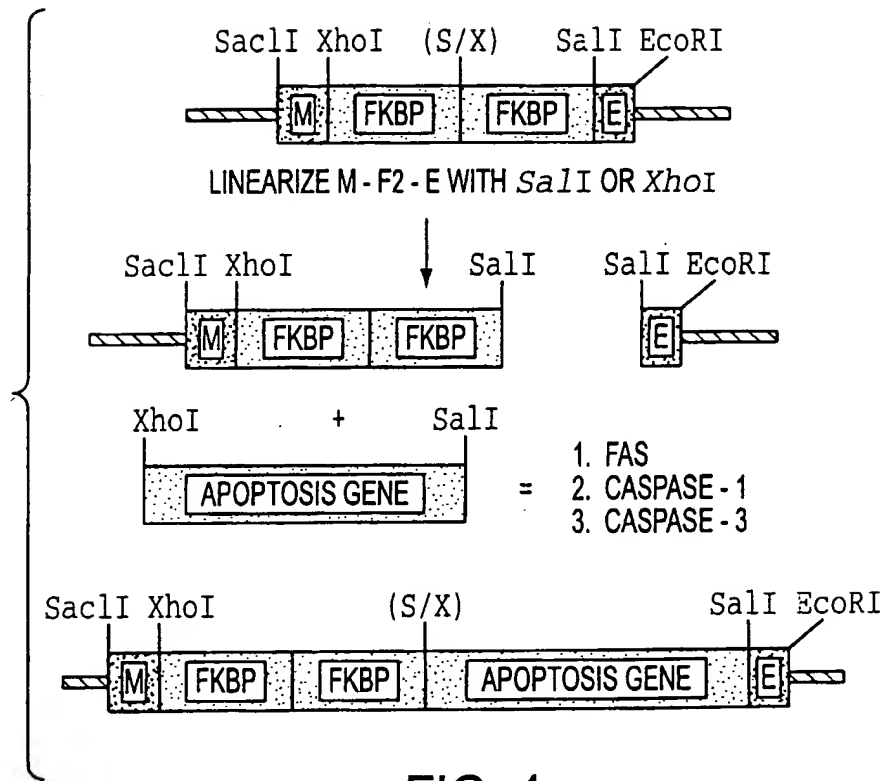


FIG. 4

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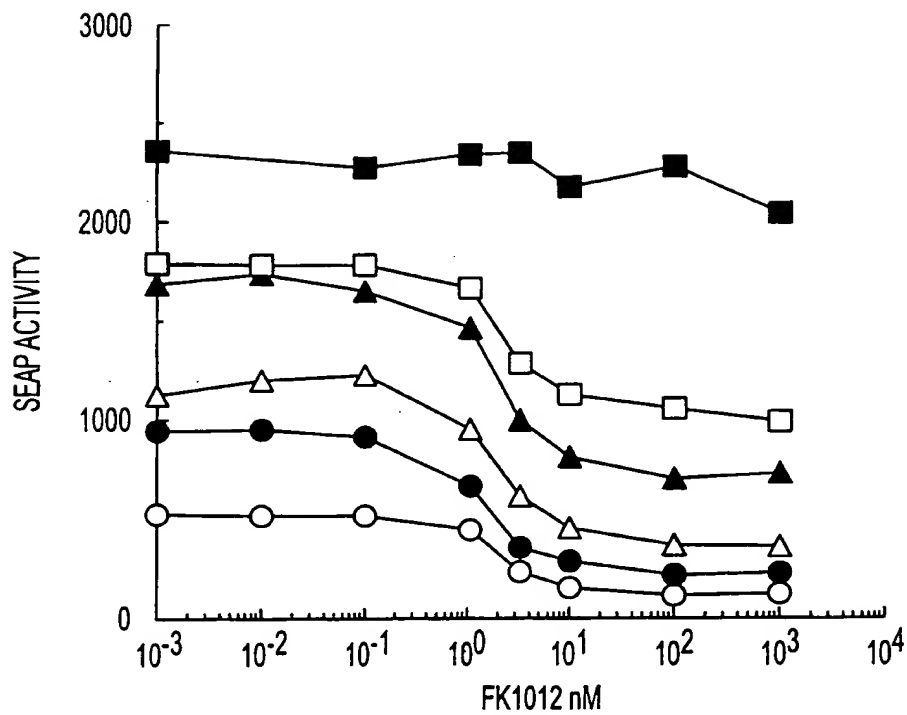


FIG. 5

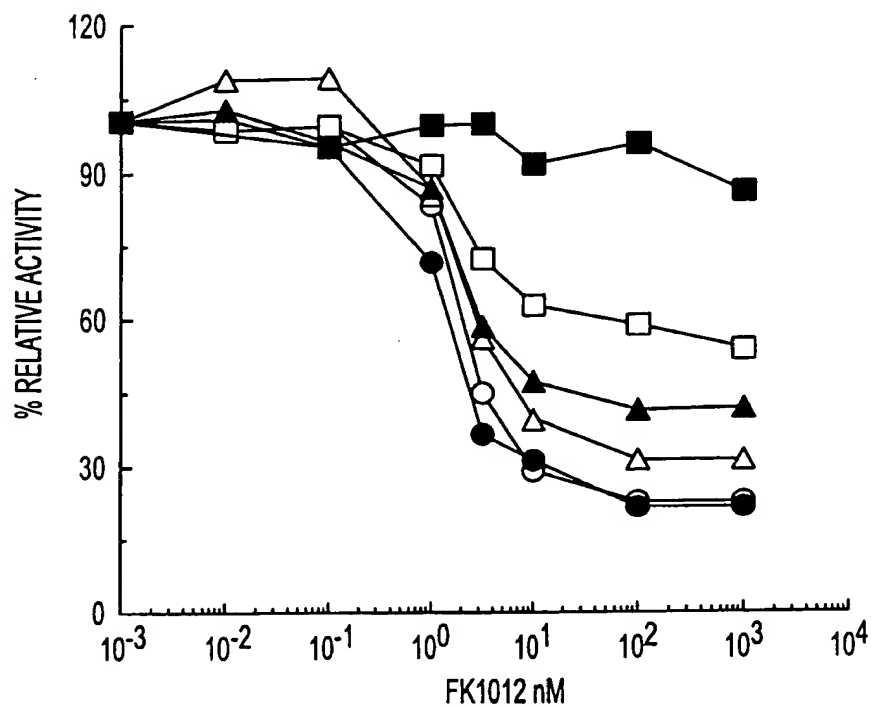


FIG. 6

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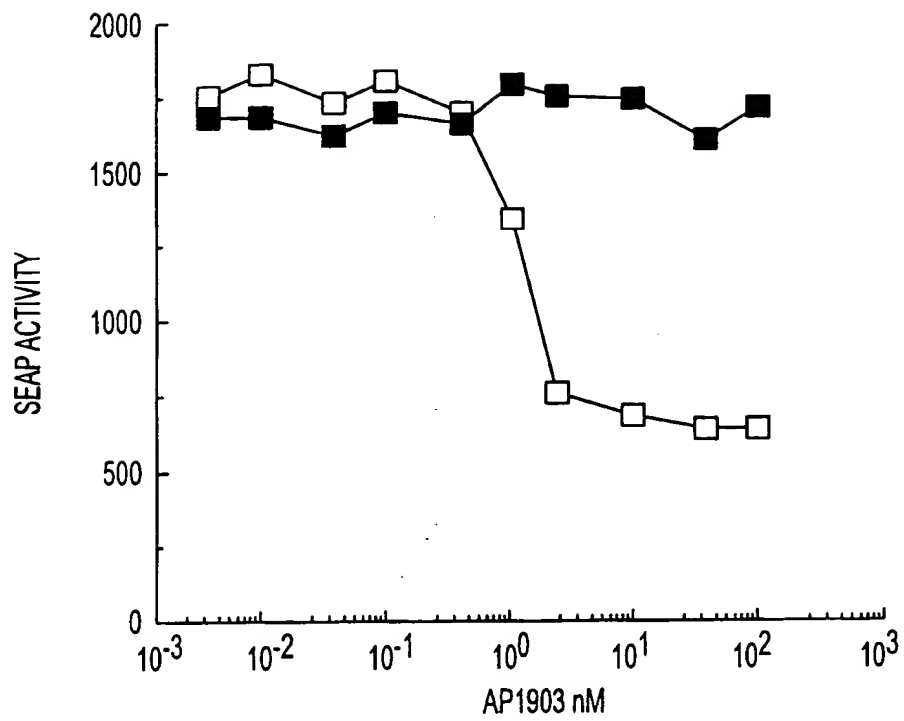


FIG. 7

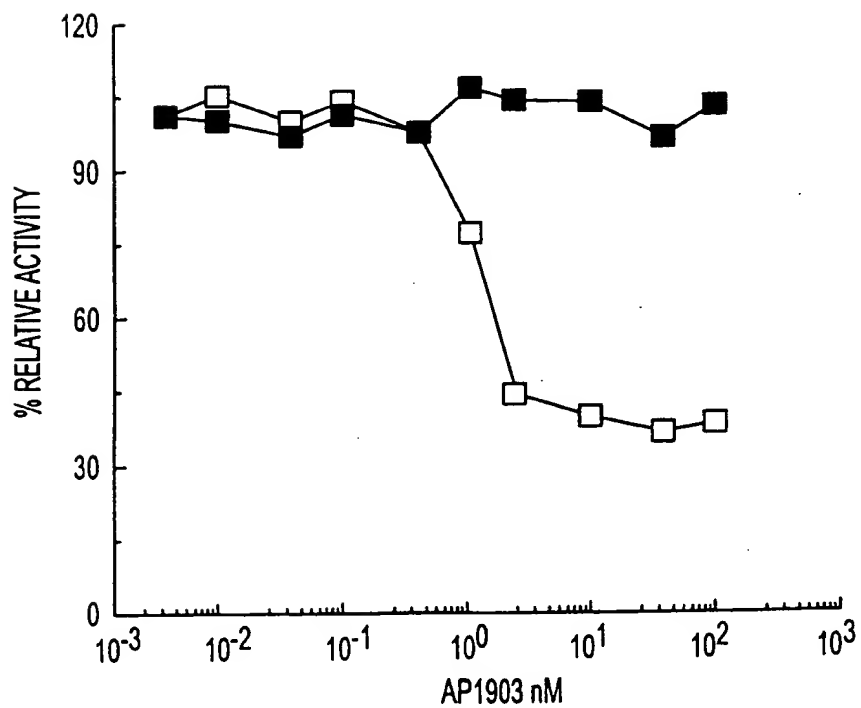


FIG. 8

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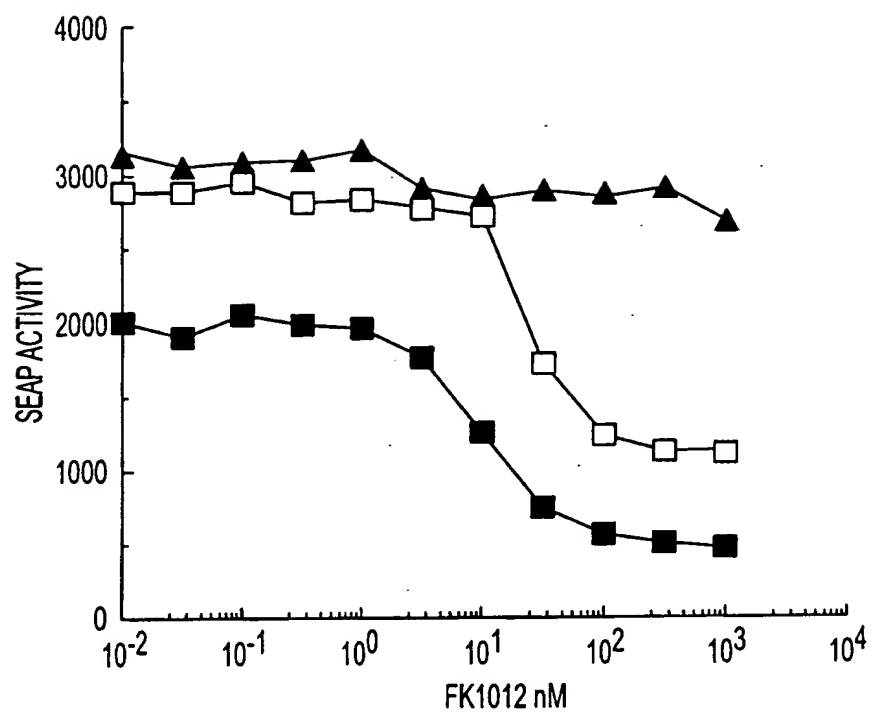


FIG. 9

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F <sub>v</sub> 2 - CASP - 3						F <sub>v</sub> 2 - CASP 3/S163					
HOURS						HOURS					
8	4	2	1	.5	0	8	4	2	1	.5	0

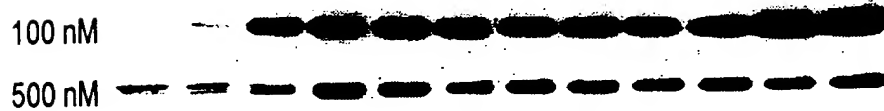


FIG. 10

F <sub>v</sub> 2 - CASP - 3						F <sub>v</sub> 2 - CASP 3/S163					
AP1903 nM						AP1903 nM					
100	32	10	3.2	1	0	100	32	10	3.2	1	0

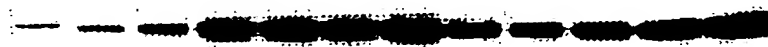


FIG. 11

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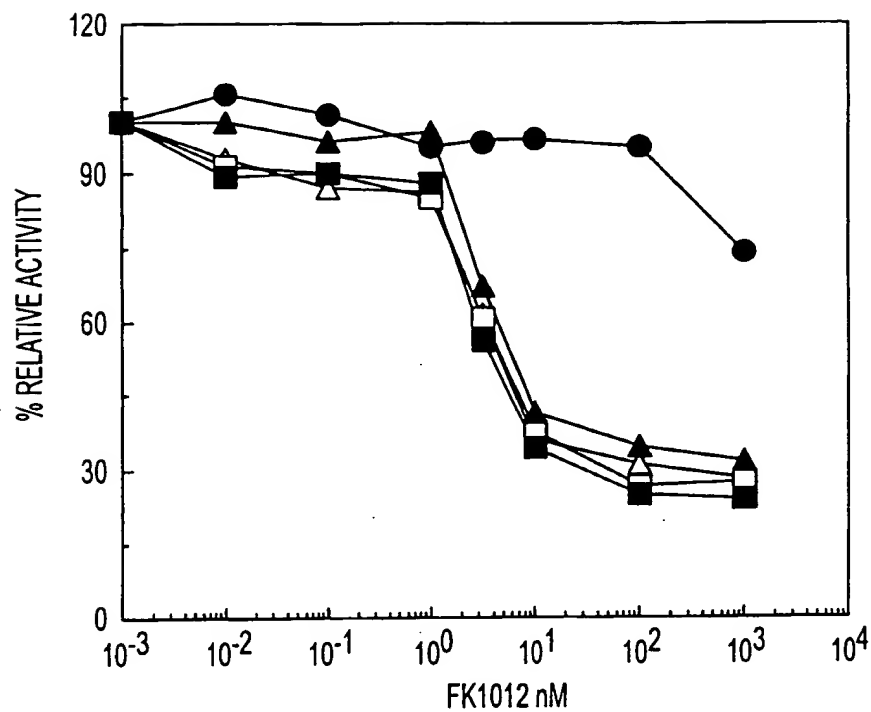


FIG. 12

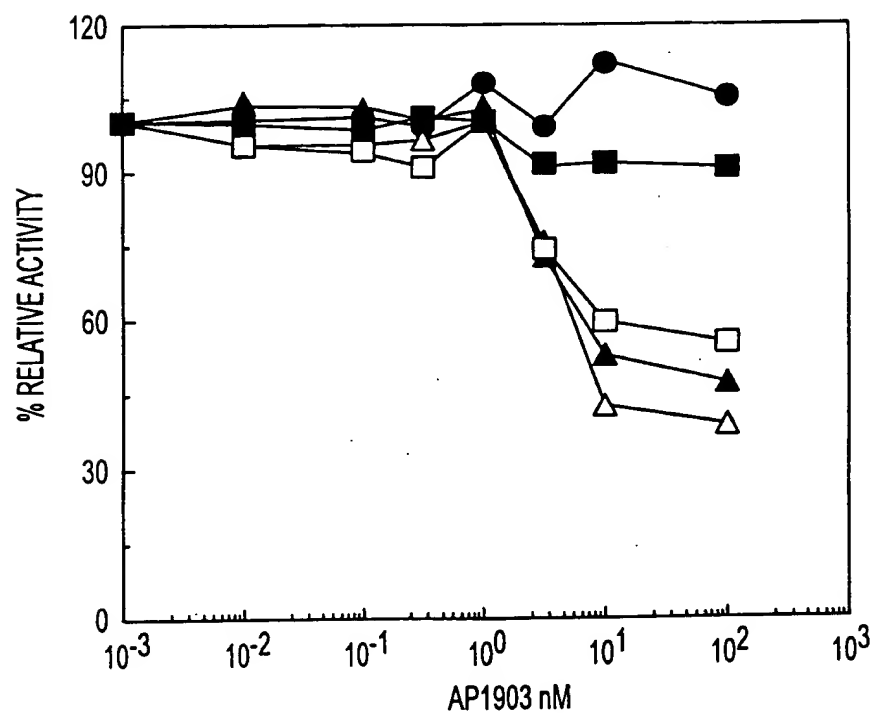


FIG. 13

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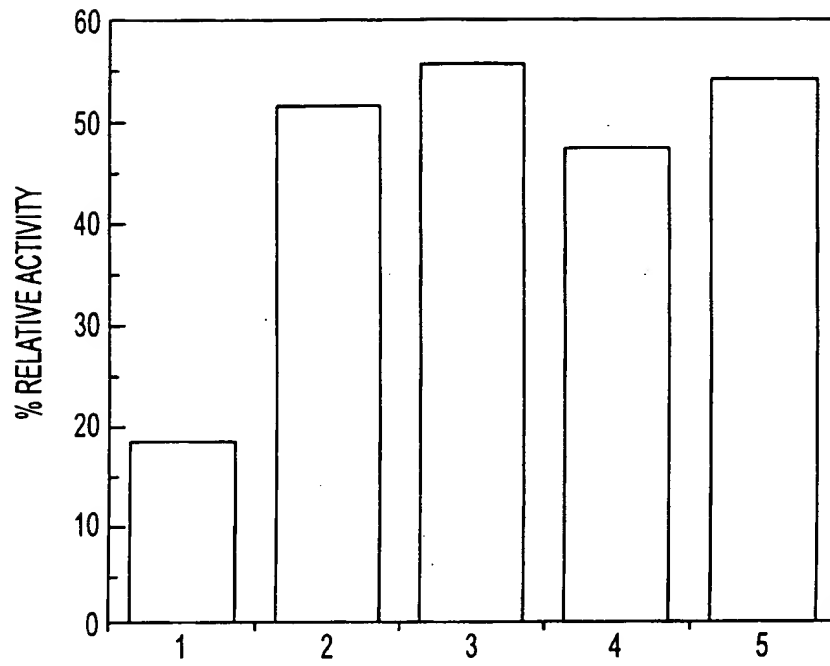


FIG. 14

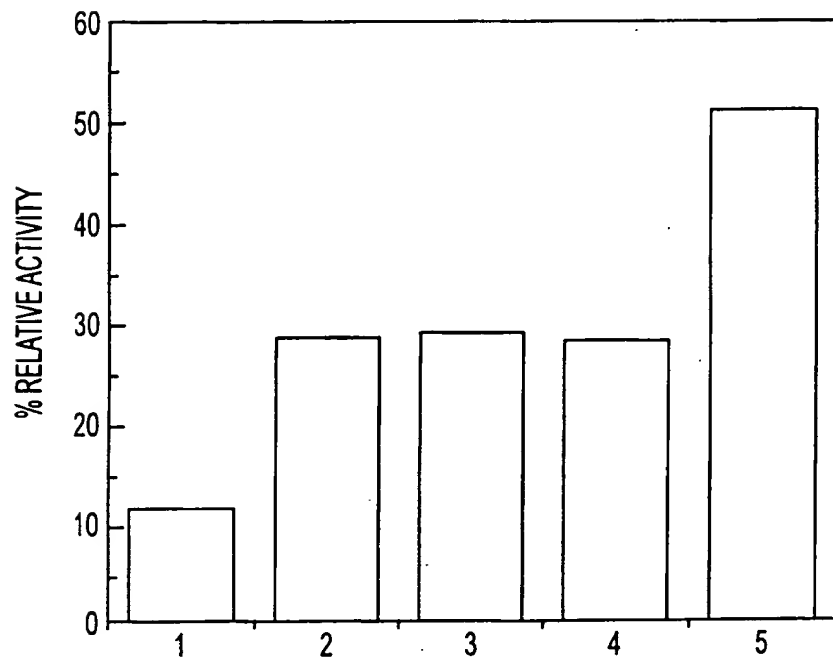


FIG. 15

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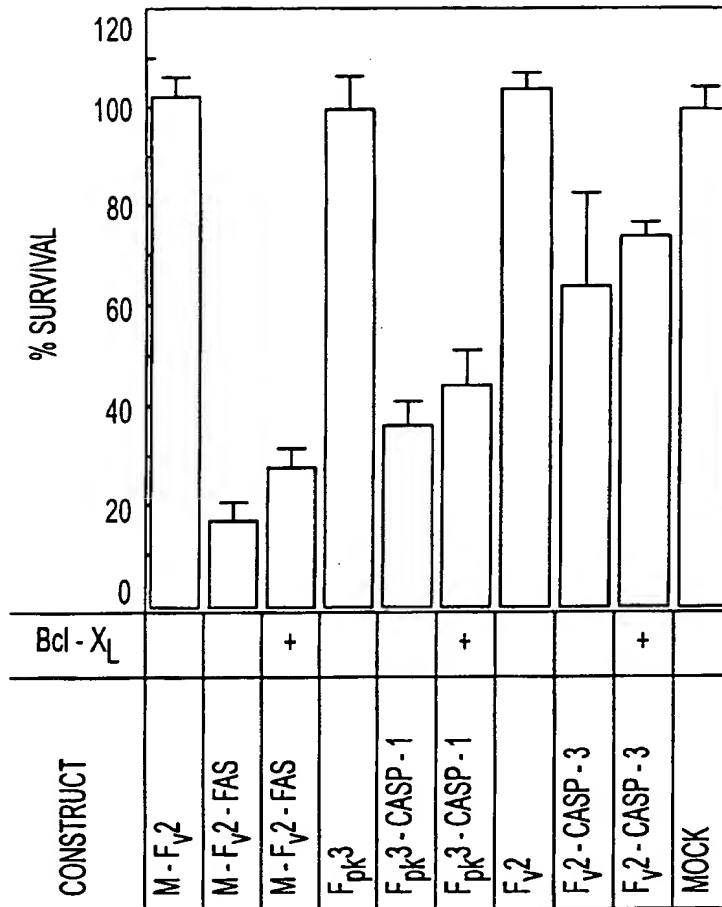


FIG. 16



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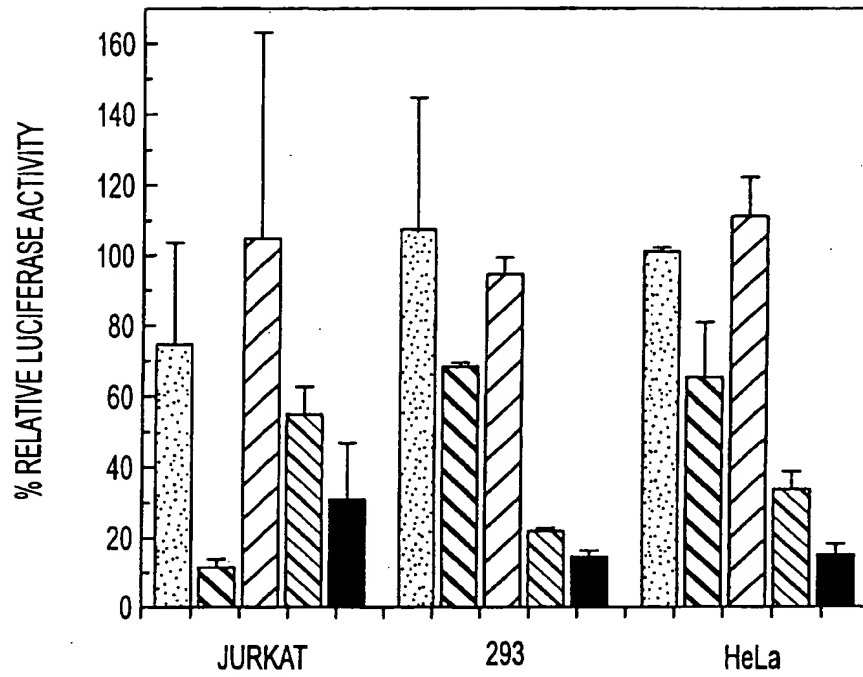


FIG. 17

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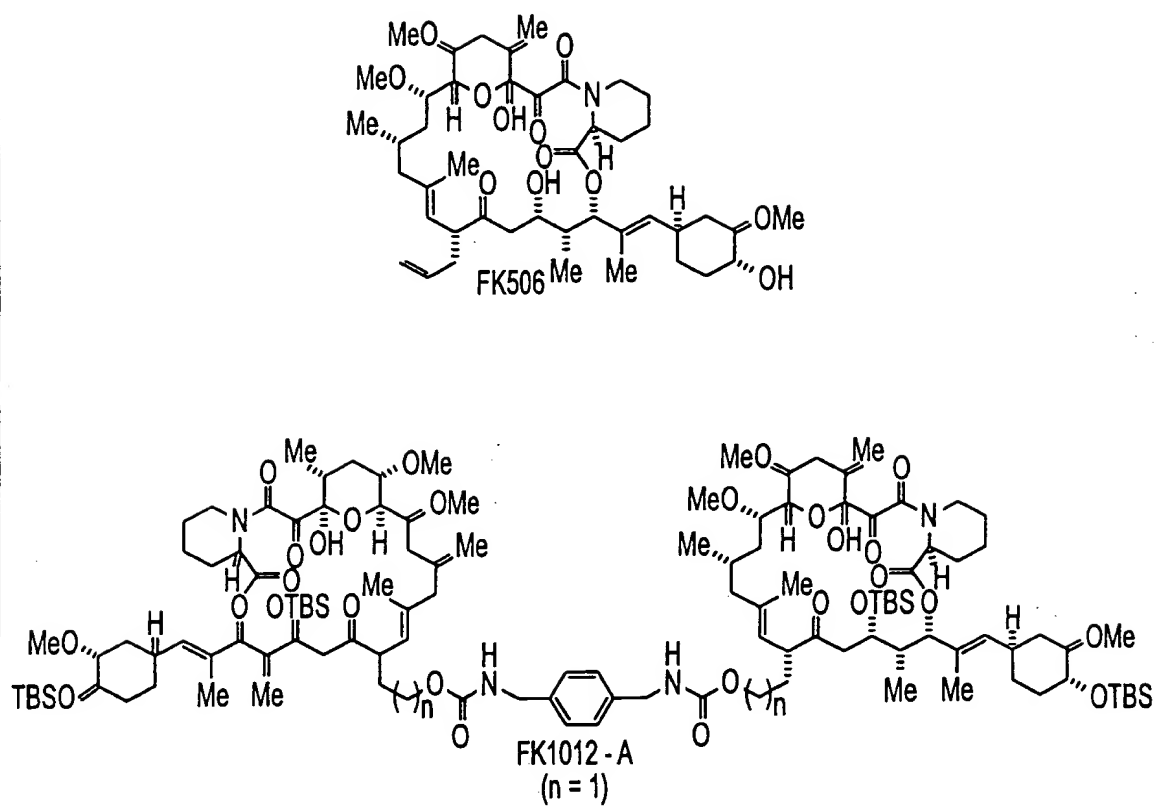


FIG. 18

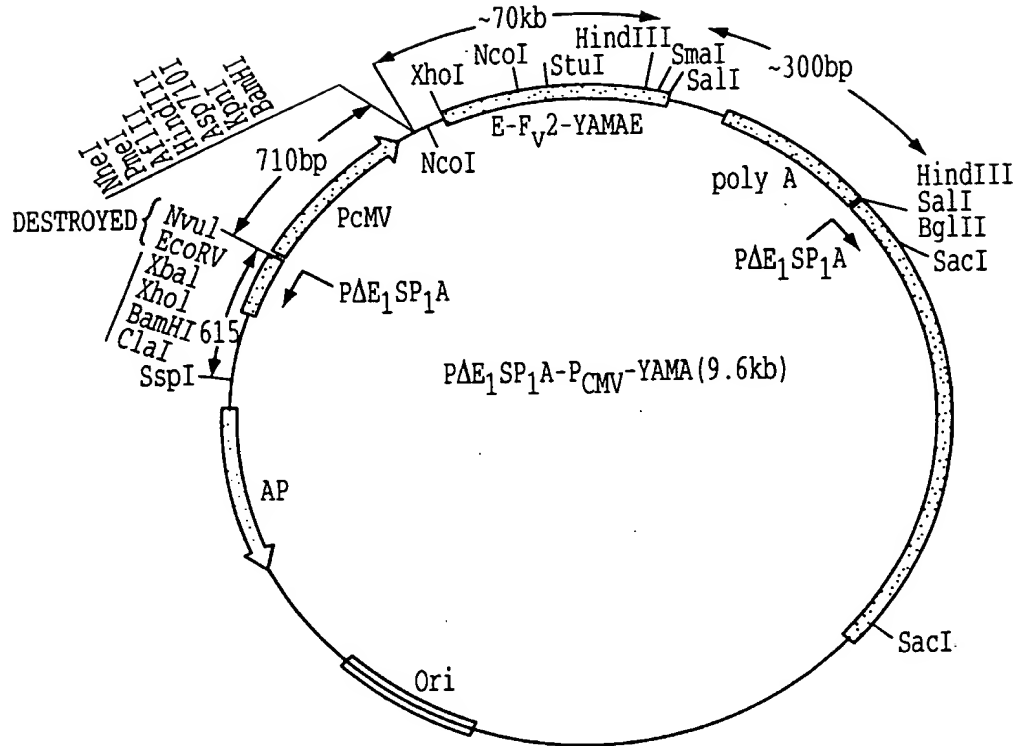


FIG. 19A



FIG. 19B

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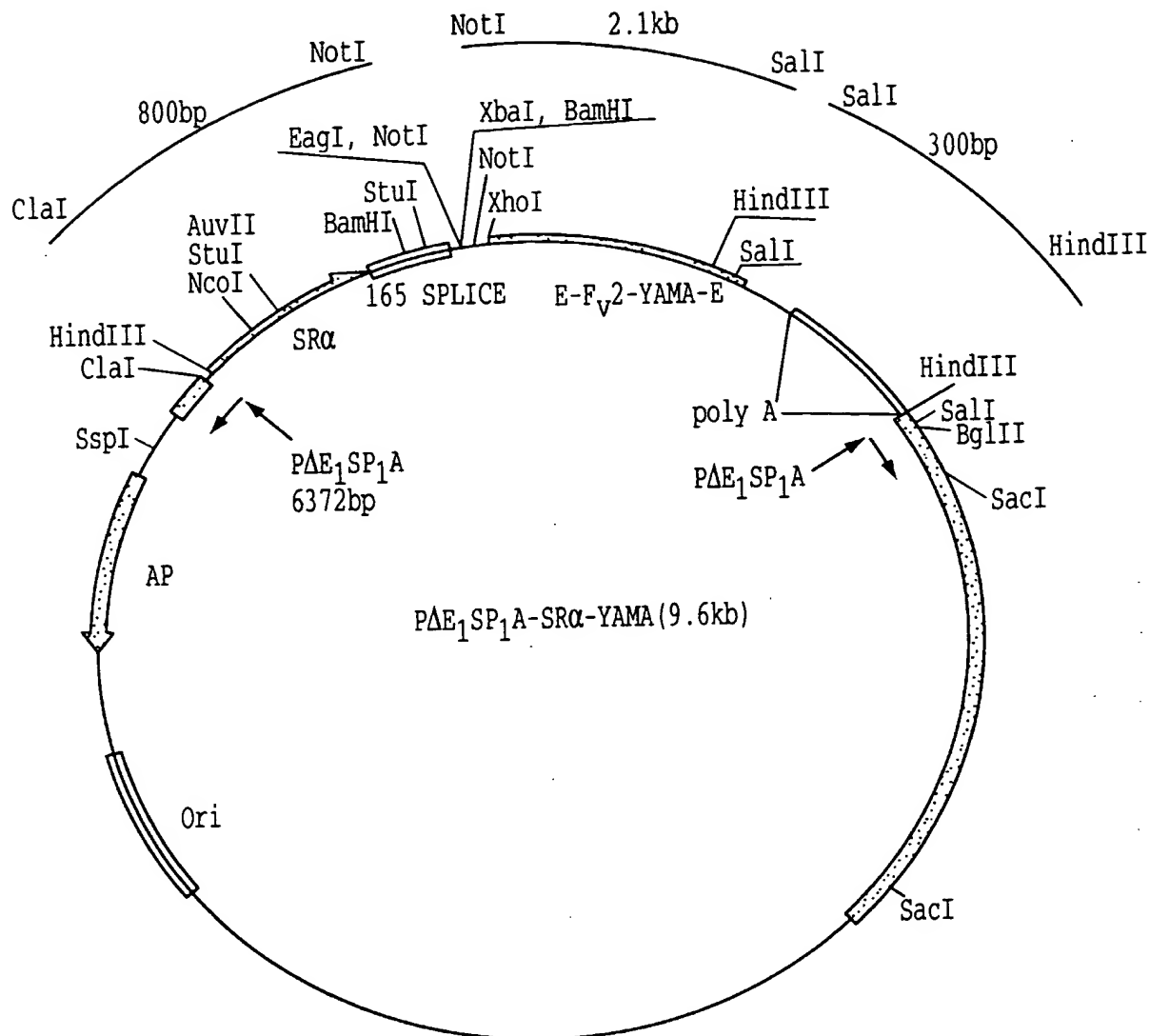


FIG. 20A

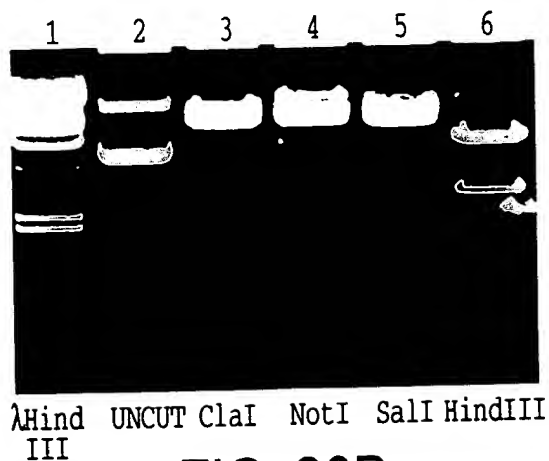


FIG. 20B

006260" 87424950

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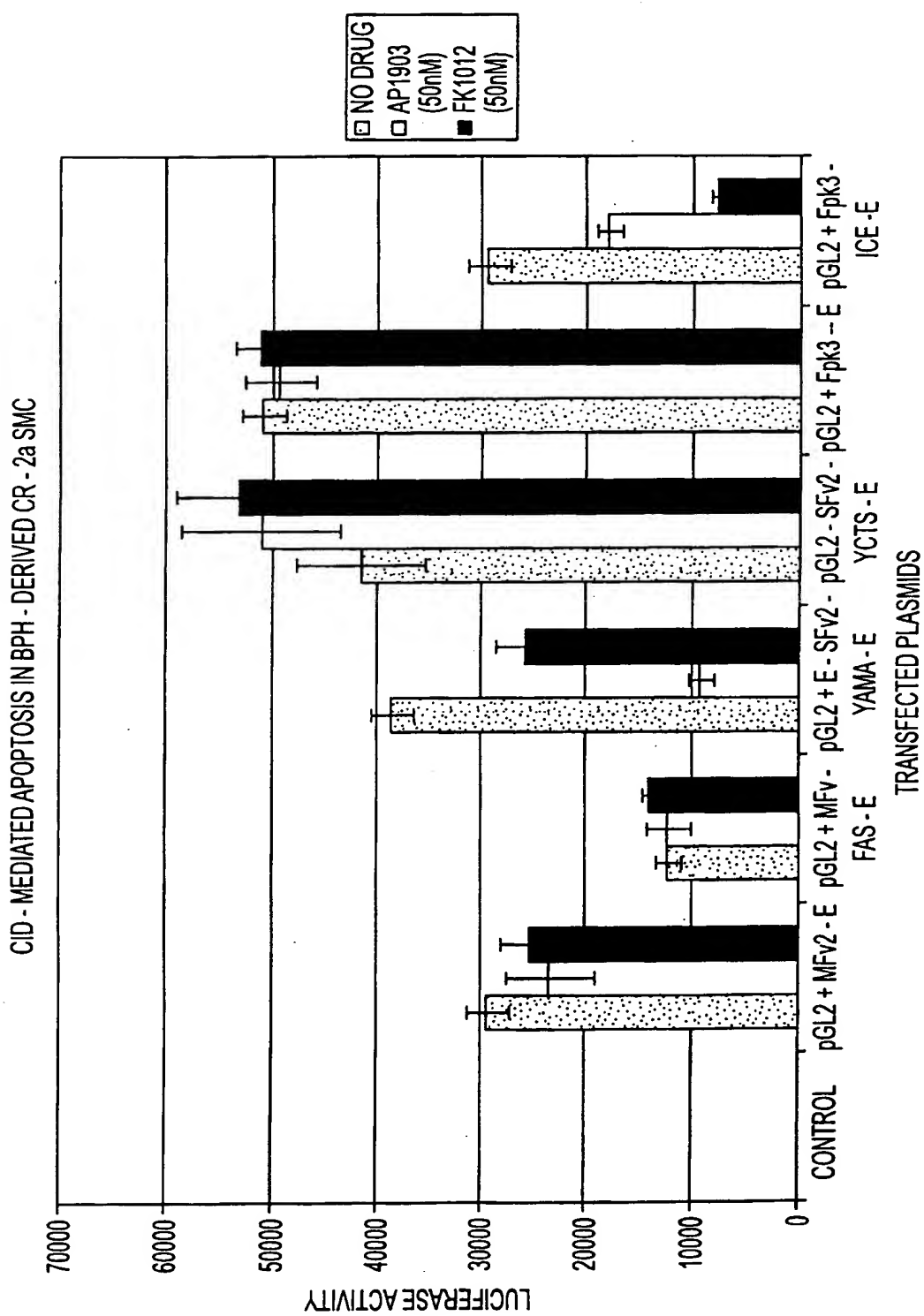


FIG. 21

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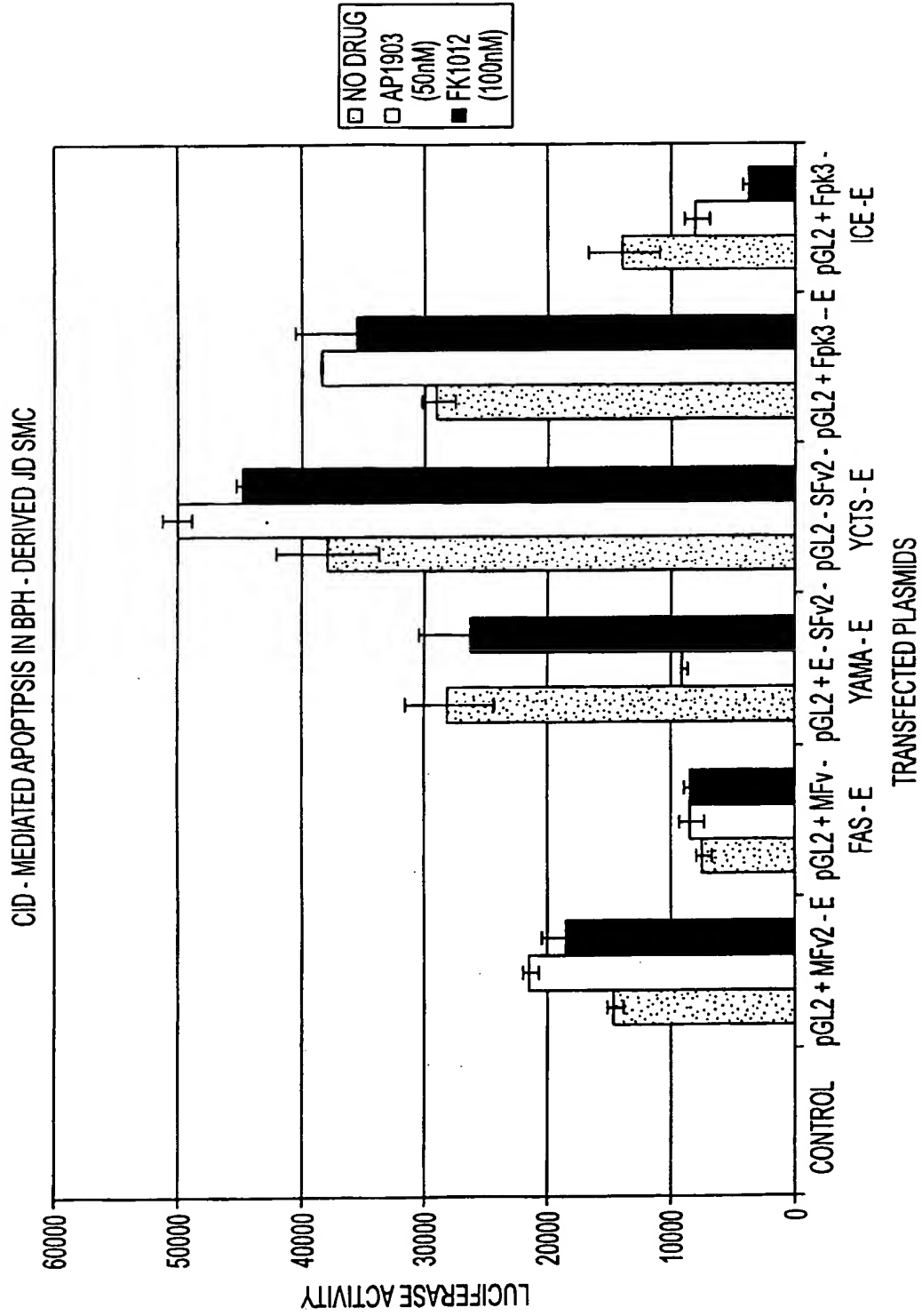


FIG. 22

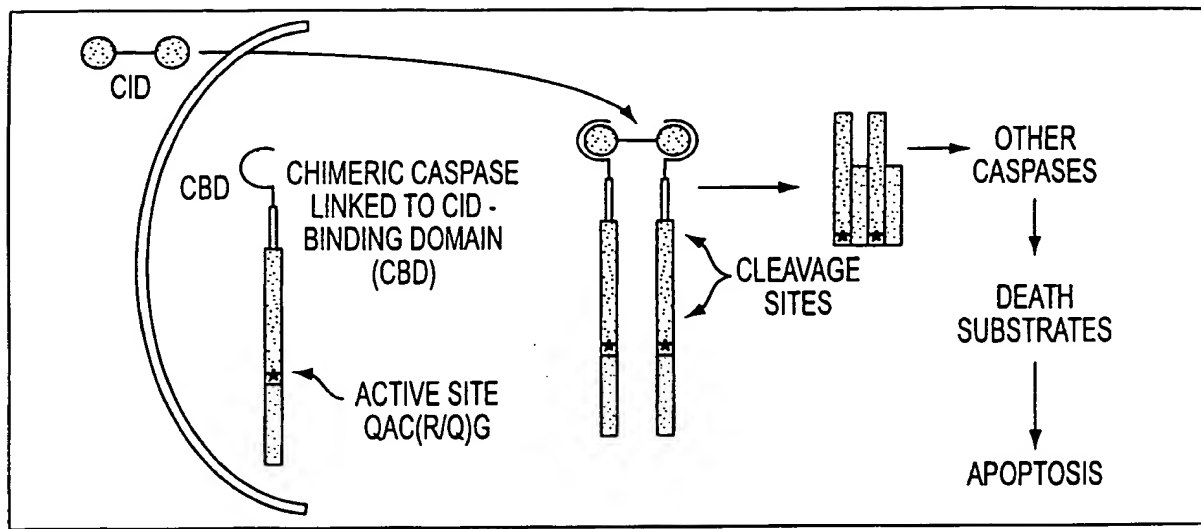


FIG. 23A

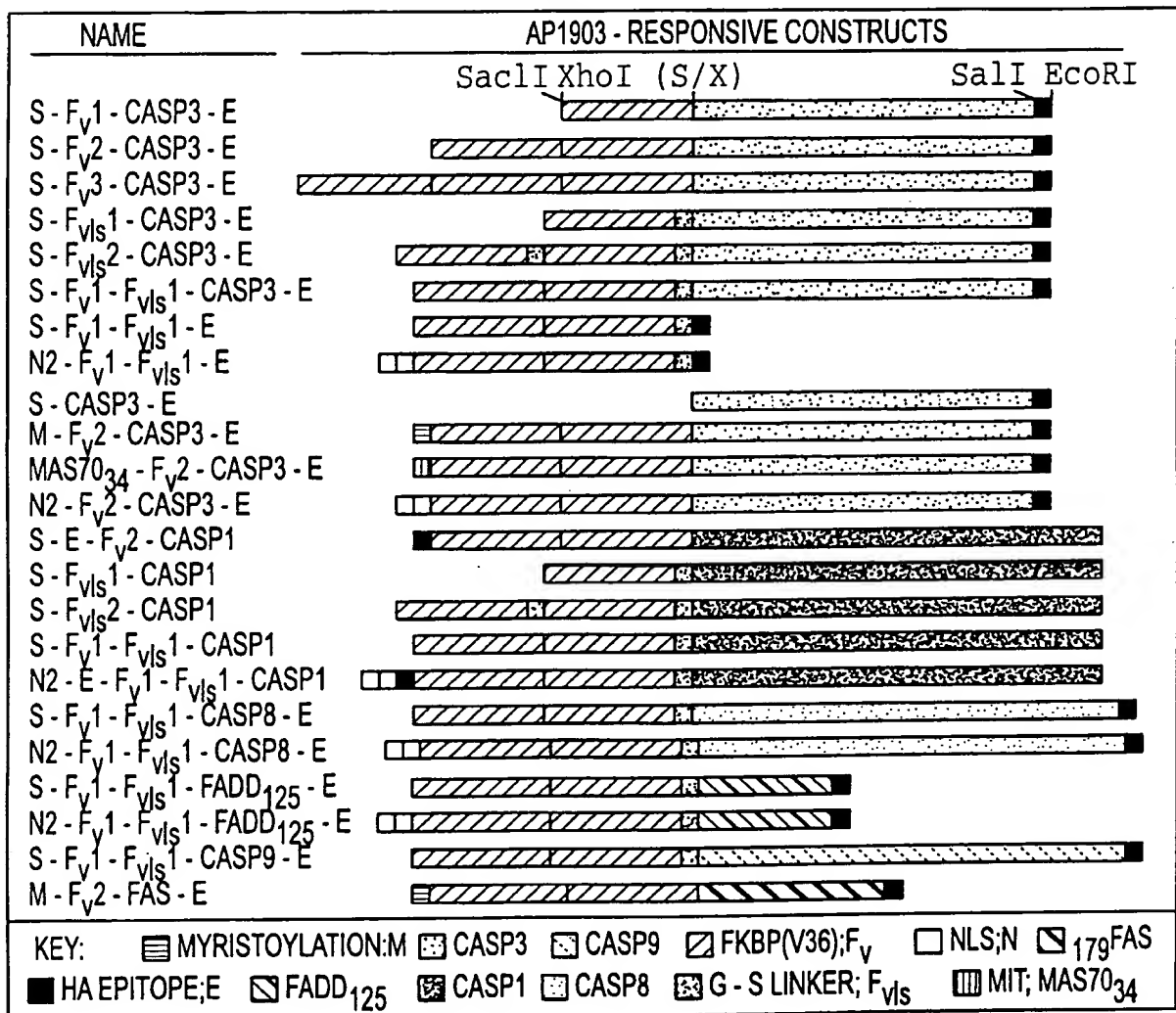


FIG. 23B

006260" 8744960

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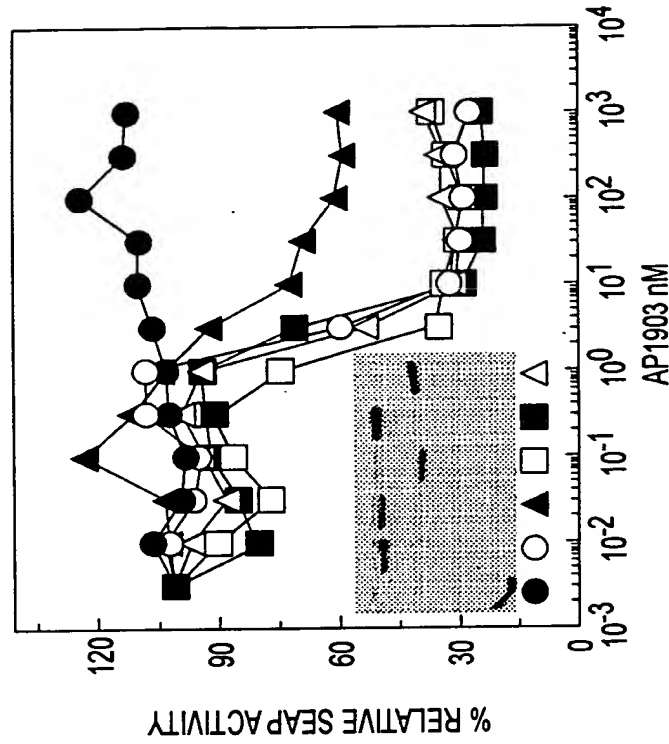


FIG. 24B

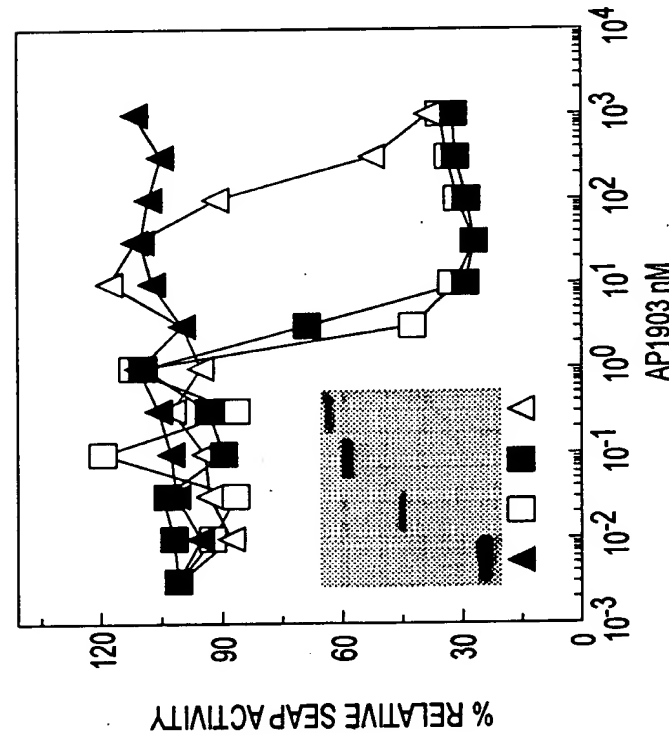


FIG. 24A



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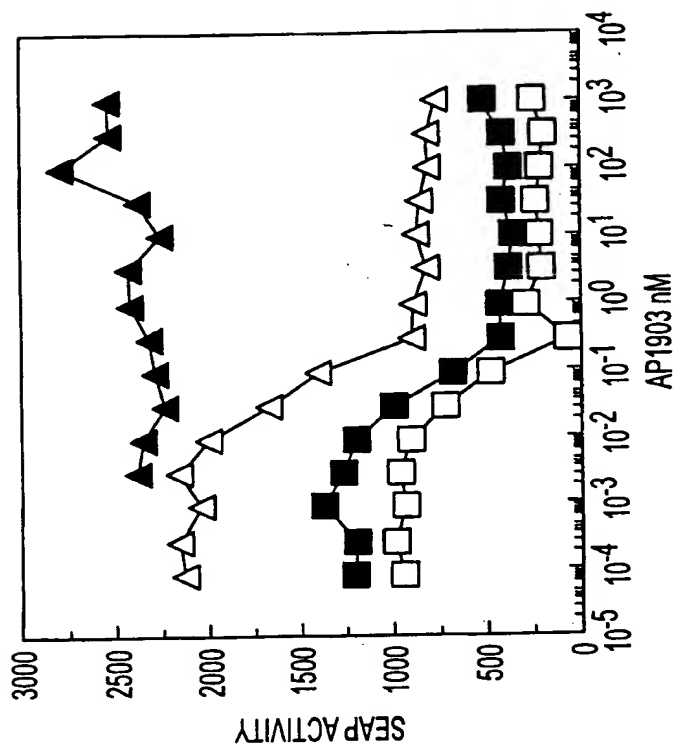


FIG. 24D

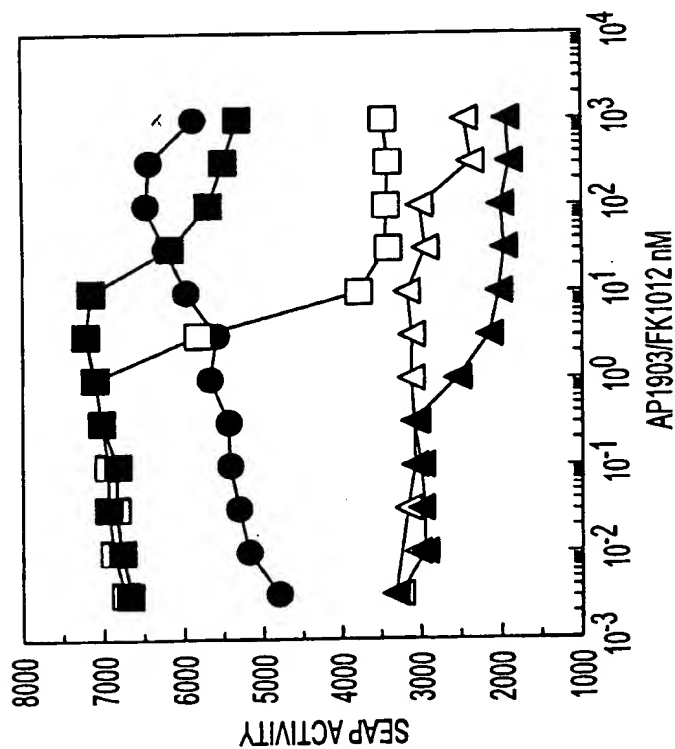


FIG. 24C

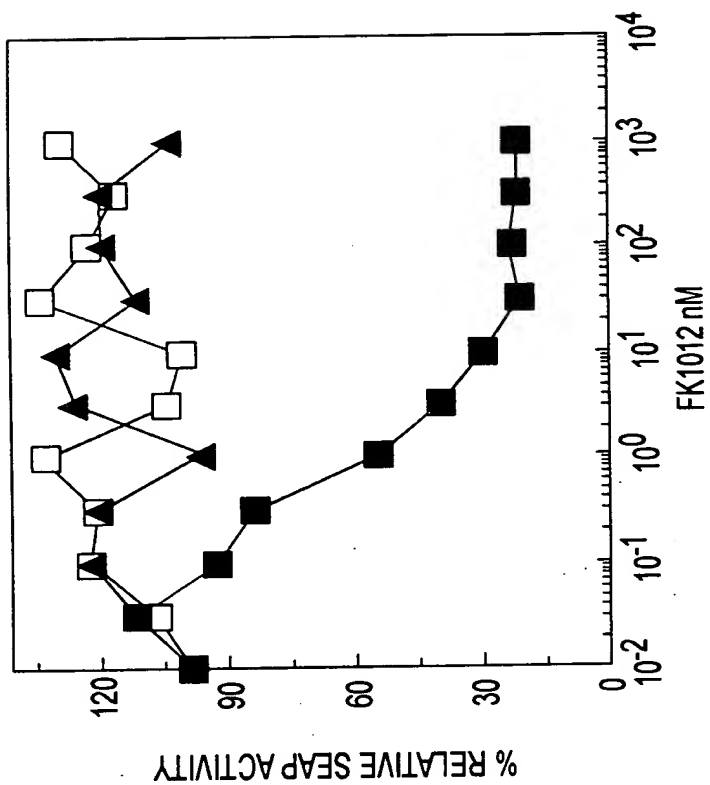


FIG. 25B

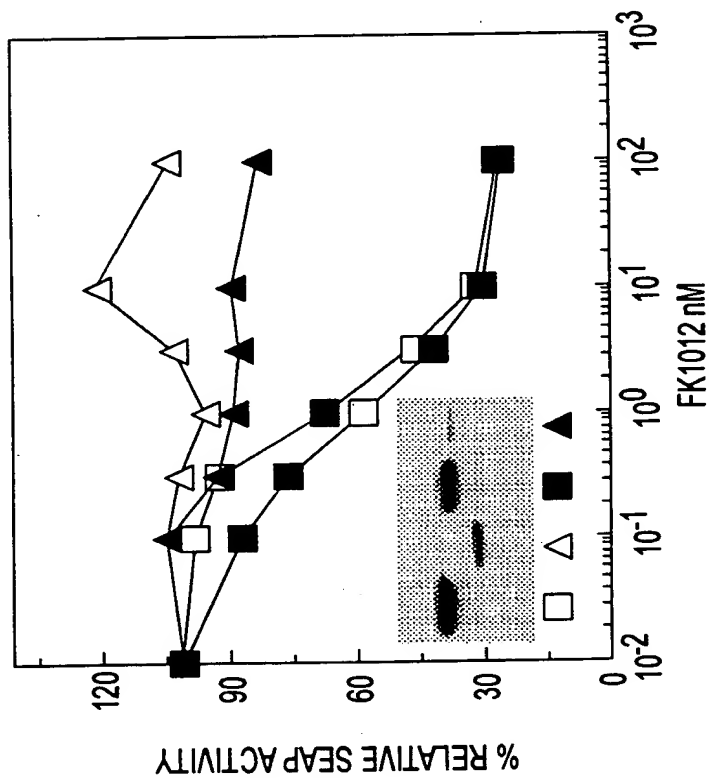


FIG. 25A

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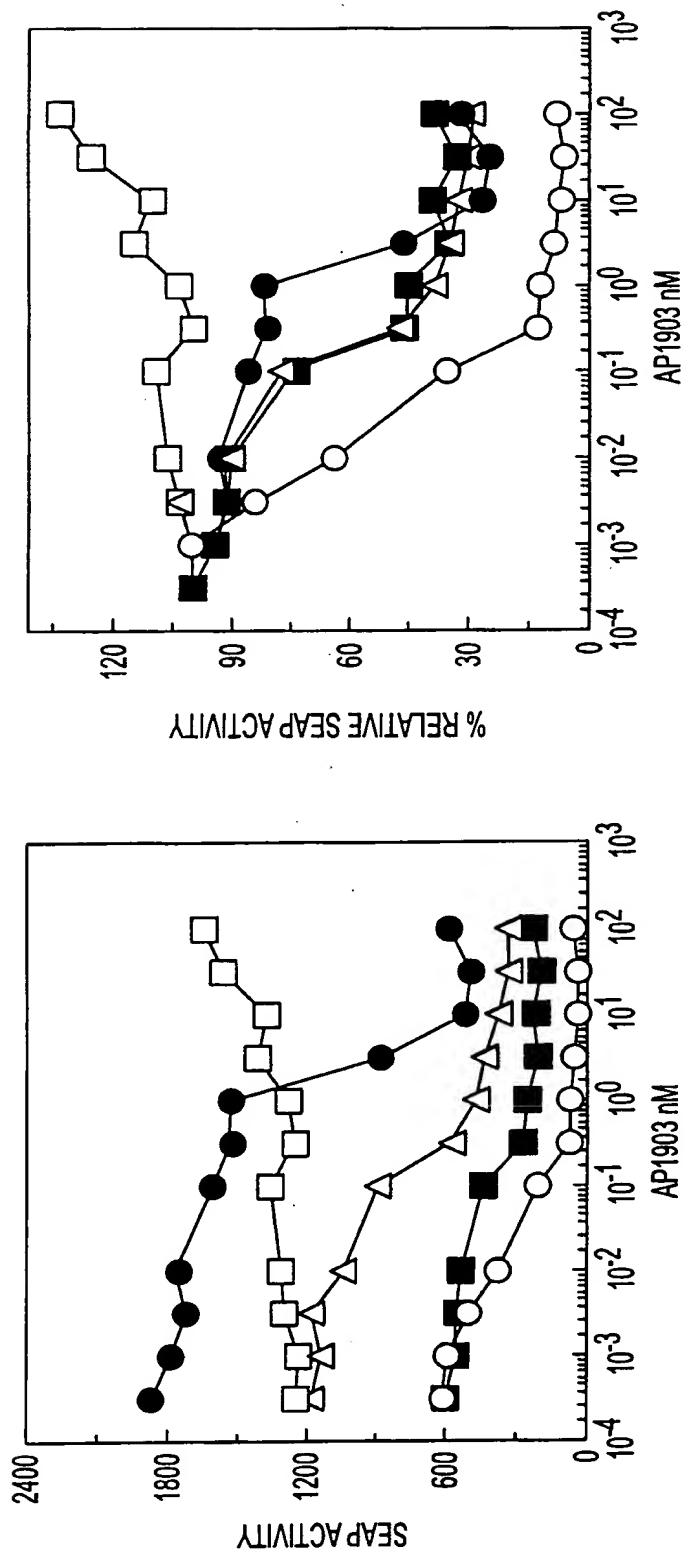


FIG. 25D

FIG. 25C

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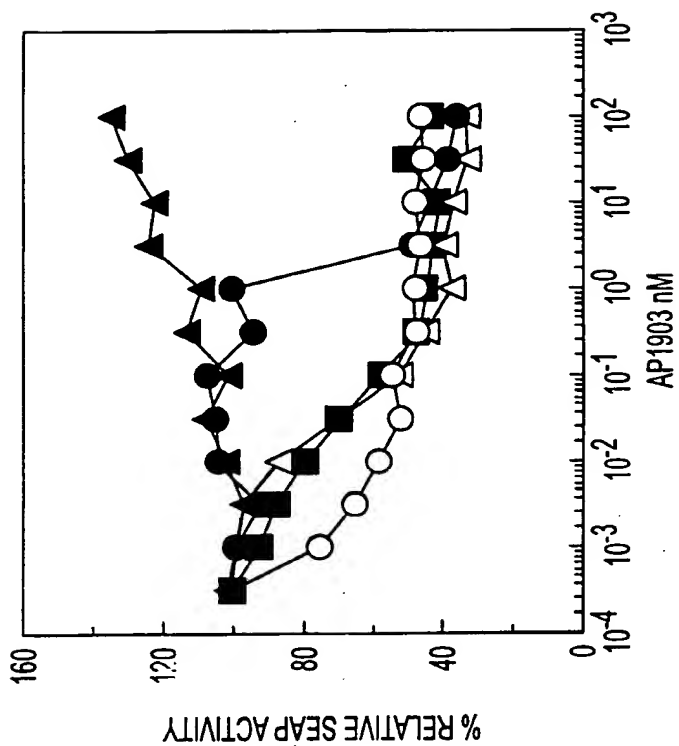


FIG. 25F

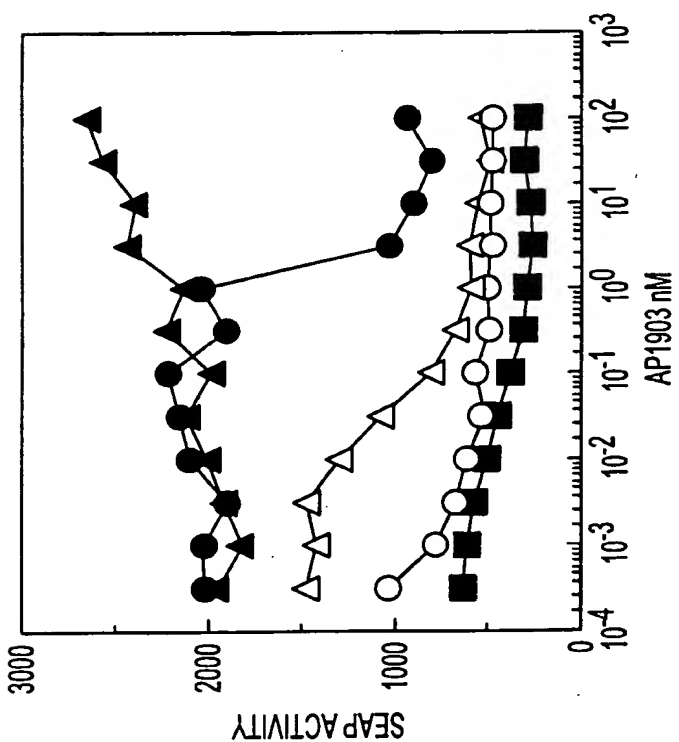


FIG. 25E

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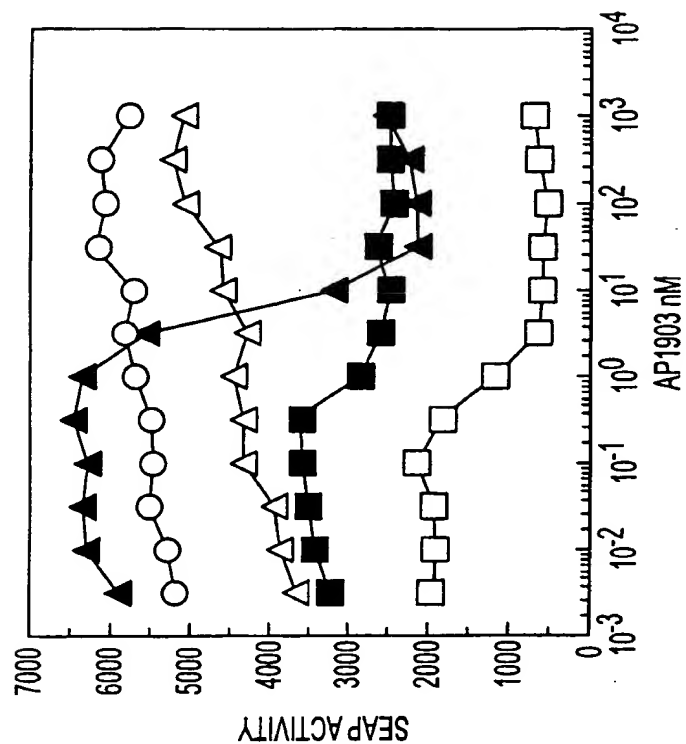


FIG. 26B

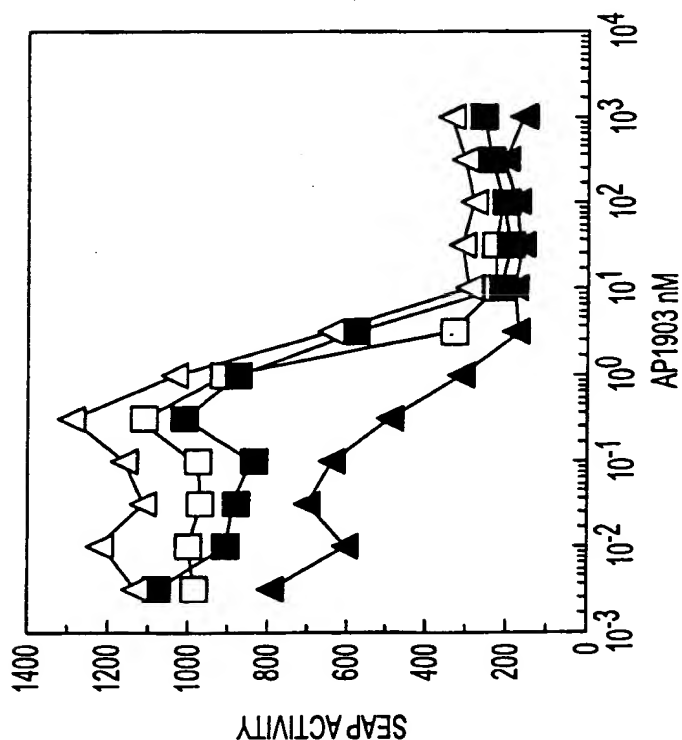


FIG. 26A

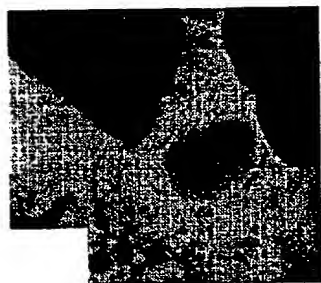


FIG. 26C



FIG. 26D

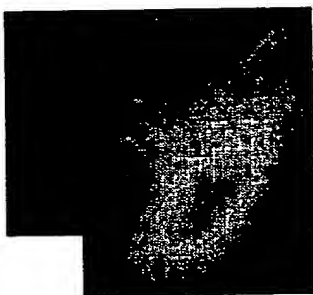


FIG. 26E

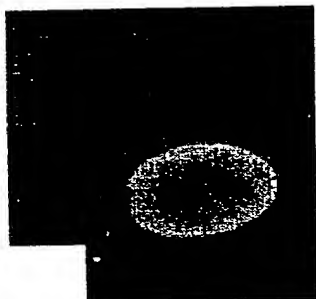


FIG. 26F

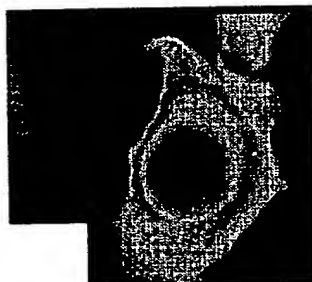


FIG. 26G

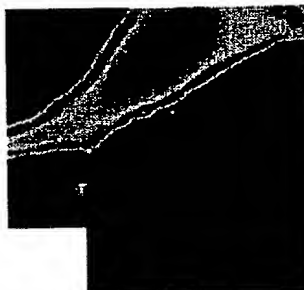


FIG. 26H



FIG. 26I

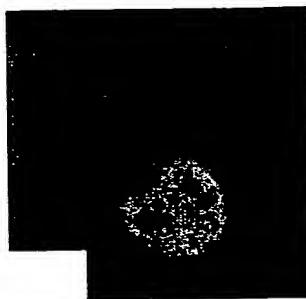


FIG. 26J

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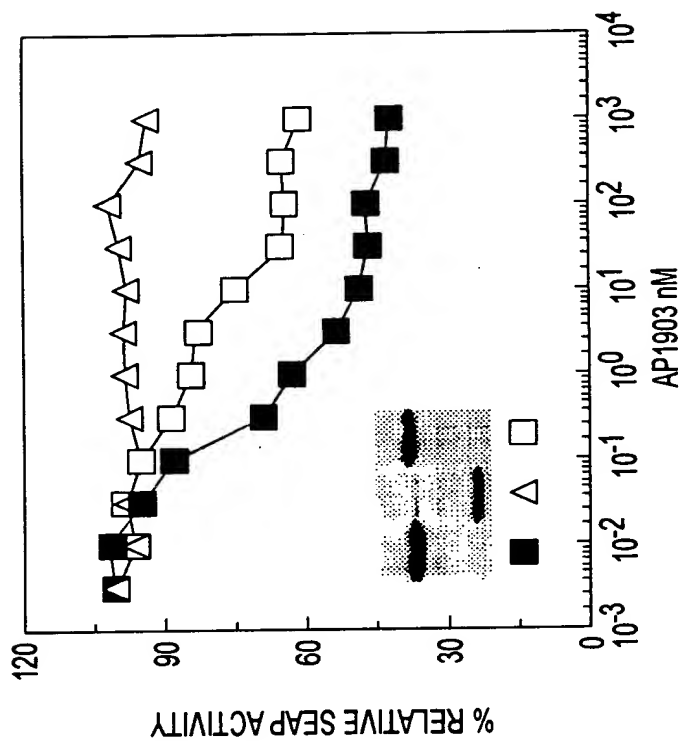


FIG. 27C

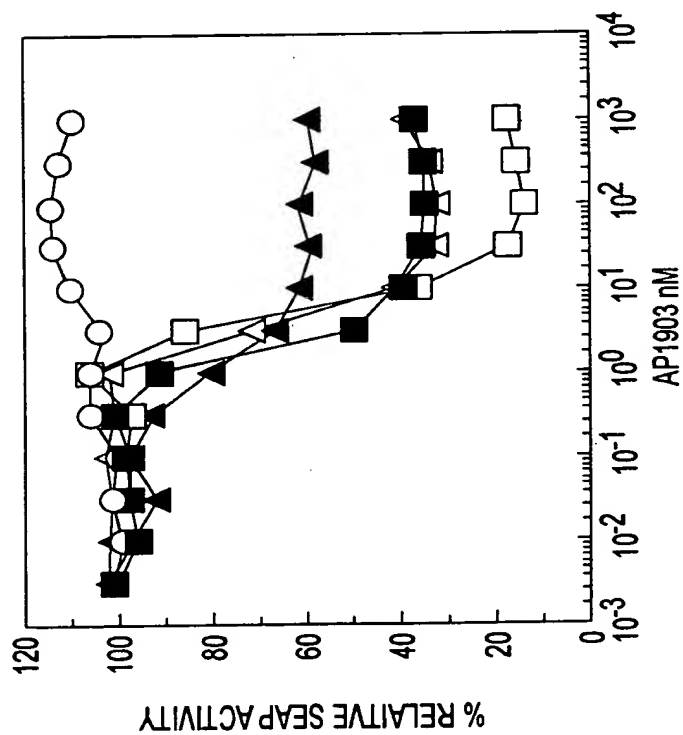


FIG. 27D

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Generation of pAd Track-CMV-F<sub>vis</sub>1-YM-E  
And pAdTrack-CMV ~E-F<sub>v</sub>1-F<sub>vis</sub>1-ICE st

1. Cut pAdTrack-CMV-with EcoRV+Not. I

1μg/2μl (pAd Track-CMV) + 1μl buffer H  
+ 1μl EcoRV + 1μl Not I + H<sub>2</sub>O 5μl  
37°C, 2 hr

2. Cut pSH1/S-F<sub>vis</sub>1-YM-E with Not I+EcoRI  
(got 2.2Kb F<sub>vis</sub>1-YM-E)  
2μg/1.2μl (pSH<sub>1</sub>/S<sub>2</sub> F<sub>vis</sub>1-YM-E+ 2μl EcoRI  
+2μl Buffer H. + H<sub>2</sub>O 14.8 μl. 37°C, 1 hr.

or

2μg/3.2μl  
12.8 H<sub>2</sub>O

Blunt

+ 10μM dNTP 12μl (10μM/each)+2μl Buffer B

14μl H<sub>2</sub>O + 4U/2μl Klenow, 25°C, 30min;+EDTA→10 min.

Inactivate Klenow by heating at 75°C, 10 min. phenol: chloroform extraction

then + 2μl Not I. 37°C, 1hr.

3. Run gel, cut bands, pool together,  
Gene Clean. Elute in 40μl

4. Ligation

Eluent 16μl + 2μl 10X ligation buffer  
+ 2μl T4 ligase, 16°C, overnight

4. Transformation

5. Miniprep with Qiagen Kit

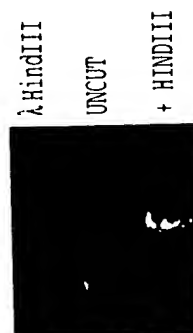


Figure 28A  
E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst

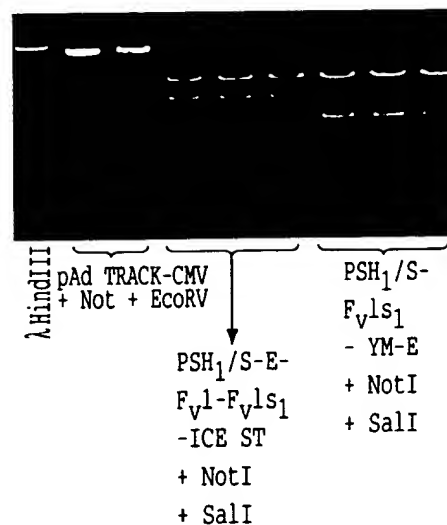


Figure 28B

005250 " 87424360



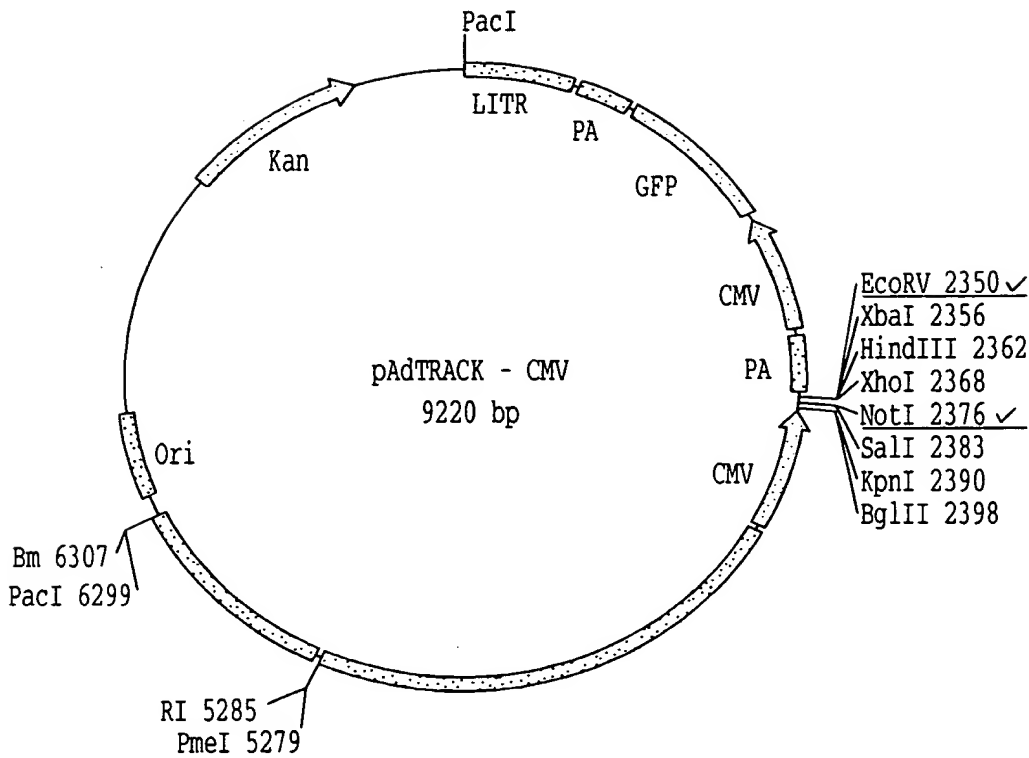


FIG. 29A

pAD TRACK - CMV - S - Fvls1 - Ym - E

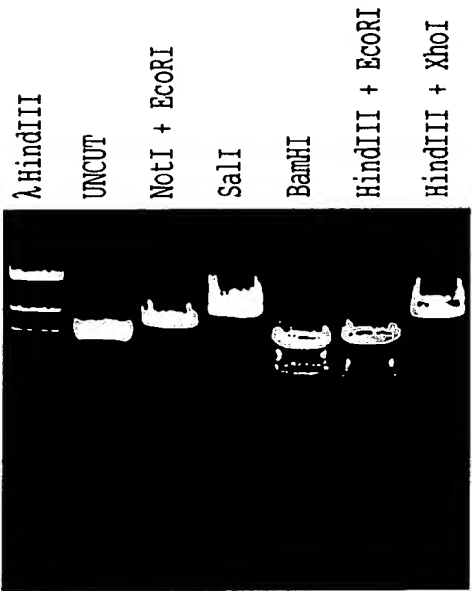


FIG. 29B



FIG. 29C

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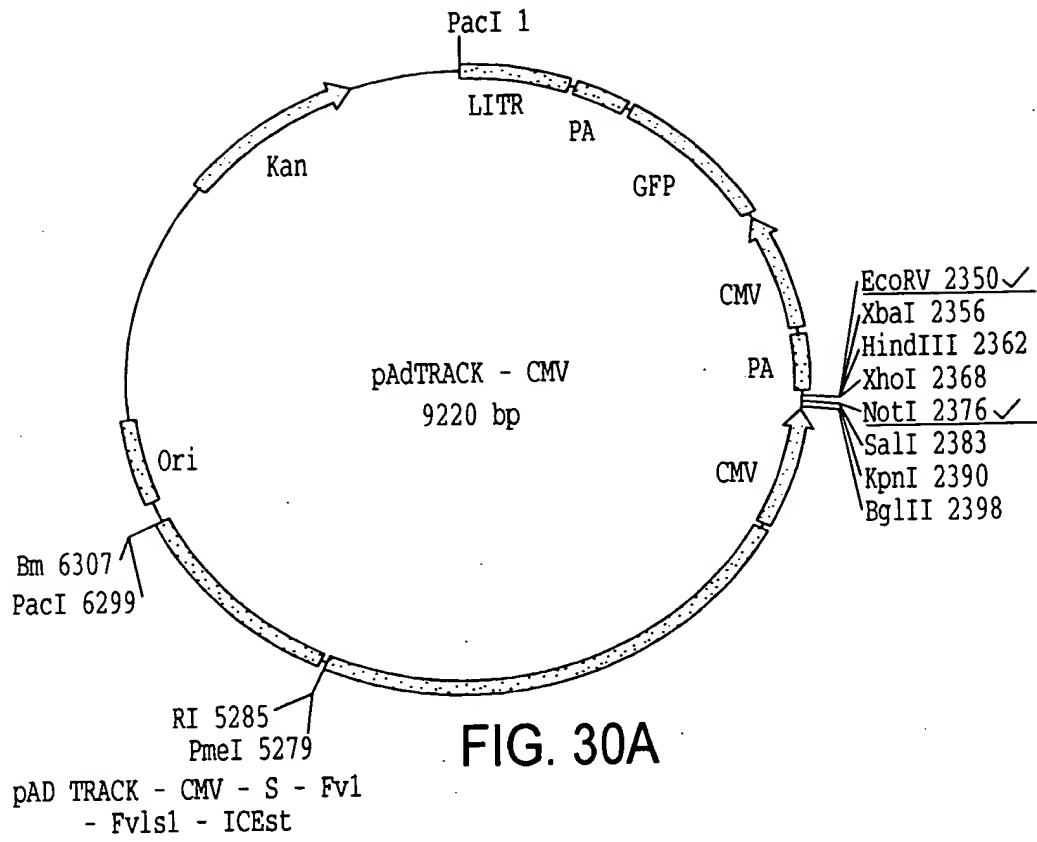


FIG. 30A

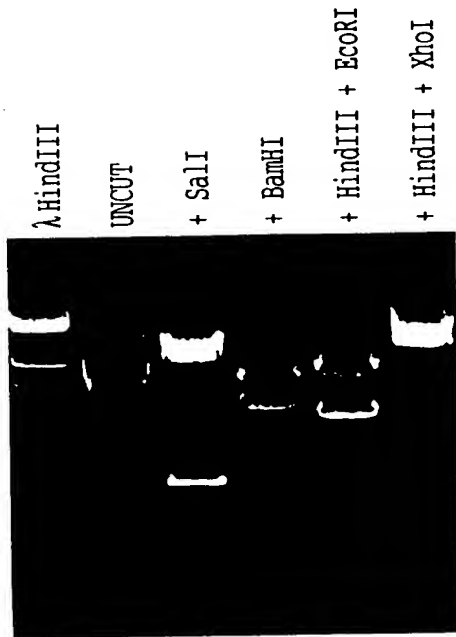


FIG. 30B

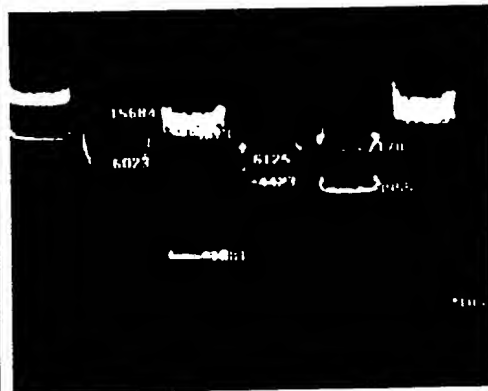


FIG. 30C



FIG. 30D

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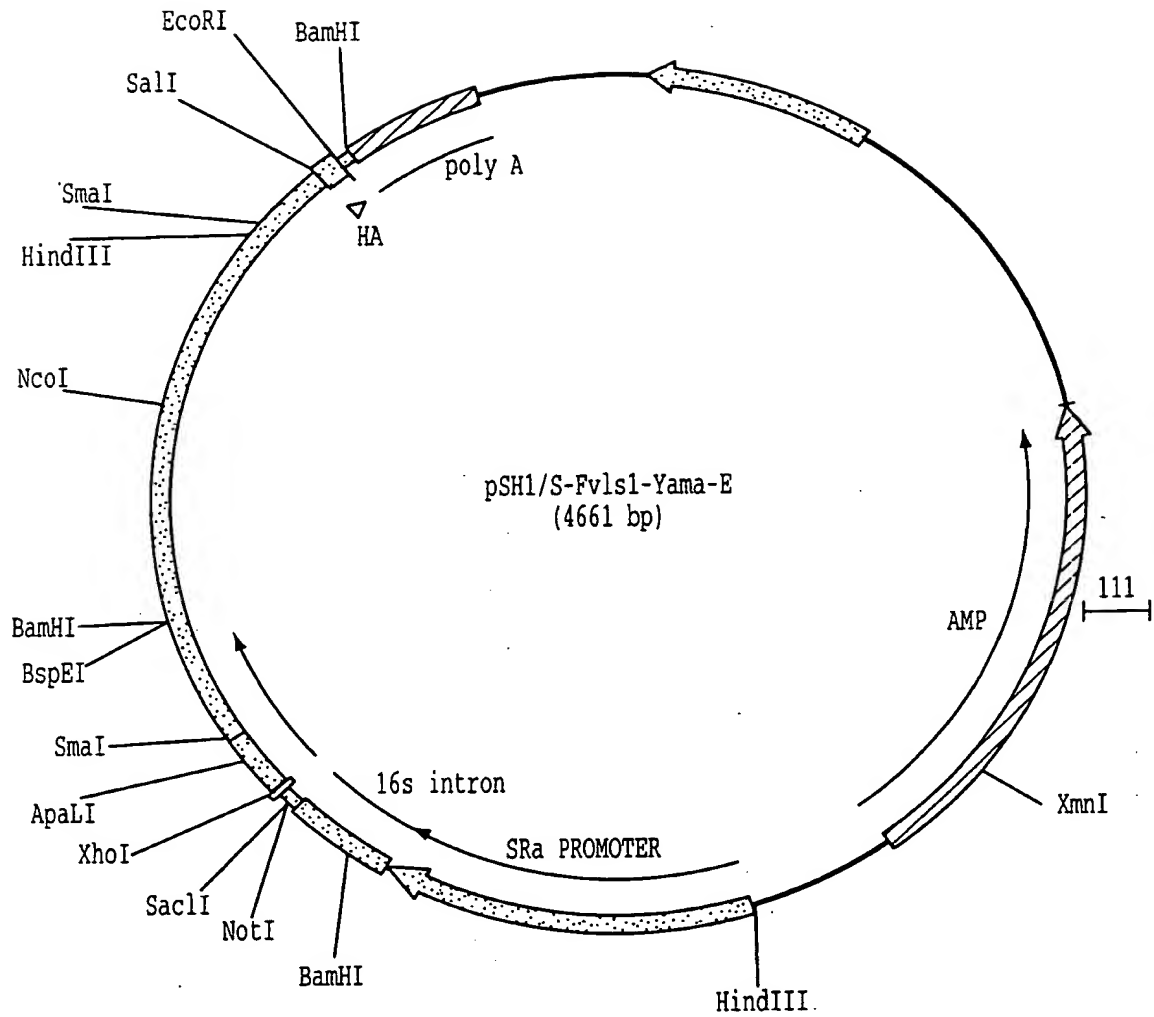


FIG. 32

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Generation of Ad-GFP-CMV-YM-E  
Ad-GFP-CMV-E-ICE

1. Co-transform E. coli BJ SH3 with pAdEasy -1 and pAdTrack-CMV-F<sub>vis</sub>1-YM-E or pAd Track-CMV-E-F<sub>vis</sub>1-F<sub>vis</sub>1-ICE st.

- a. Linearize the shuttle plasmids with PmeI  
1 $\mu$ g of pAdTrack-CMV-F<sub>vis</sub>1-YM-E  
or  
1 $\mu$ g of pAdTrack-CMV-E-F<sub>vis</sub>1-F<sub>vis</sub>1-ICEst  
+ 1 $\mu$ l Buffer + H<sub>2</sub>O  $\rightarrow$  9 $\mu$ l, + 1 $\mu$ l PmeI

37°C, 2hr

phenol-chloroform extraction, ethanol precipitation and resuspend in 6 $\mu$ l H<sub>2</sub>O

- b. Co-transformation: with 100ng pAdEasy -1  
20 $\mu$ l of BJ5183  
2.0mm cuvettes  
at 2,500V, 200 Ohms, 25  $\mu$ FD
- c. Selection:  
pick 10 smallest colonies, miniprep. check with PacI.  
Retransform-XL-1 Blue with the correct plasmid, miniprep, recheck  
with Pac I. Midiprep.
- d. Transfect 293 cells with FuGene.

90% confluence 293 cells in G-well-plate  
4 $\mu$ g plasmid/6 $\mu$ l FuGene/well

Figure 33

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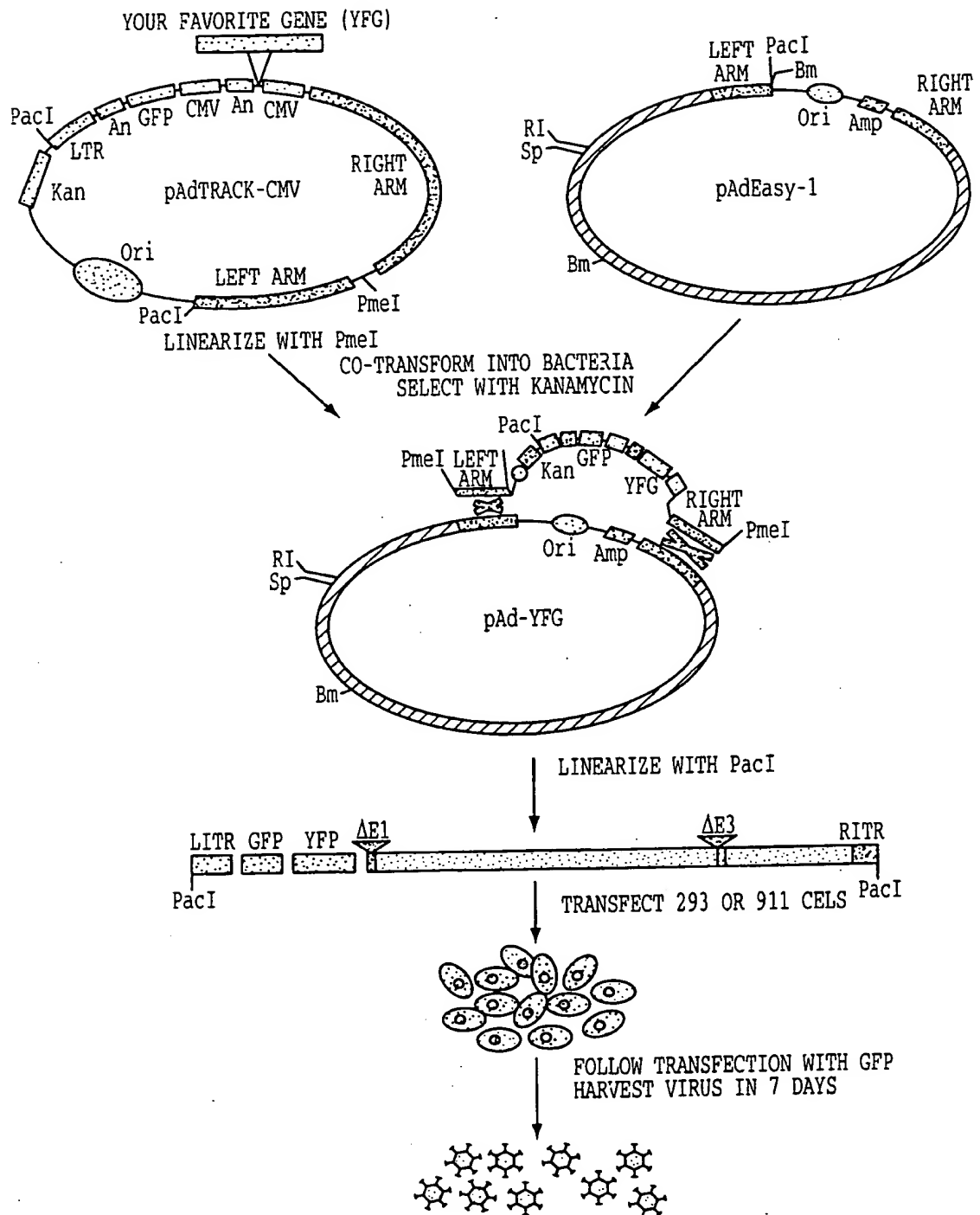


FIG. 34

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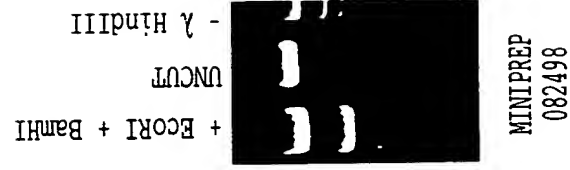


FIG. 35B

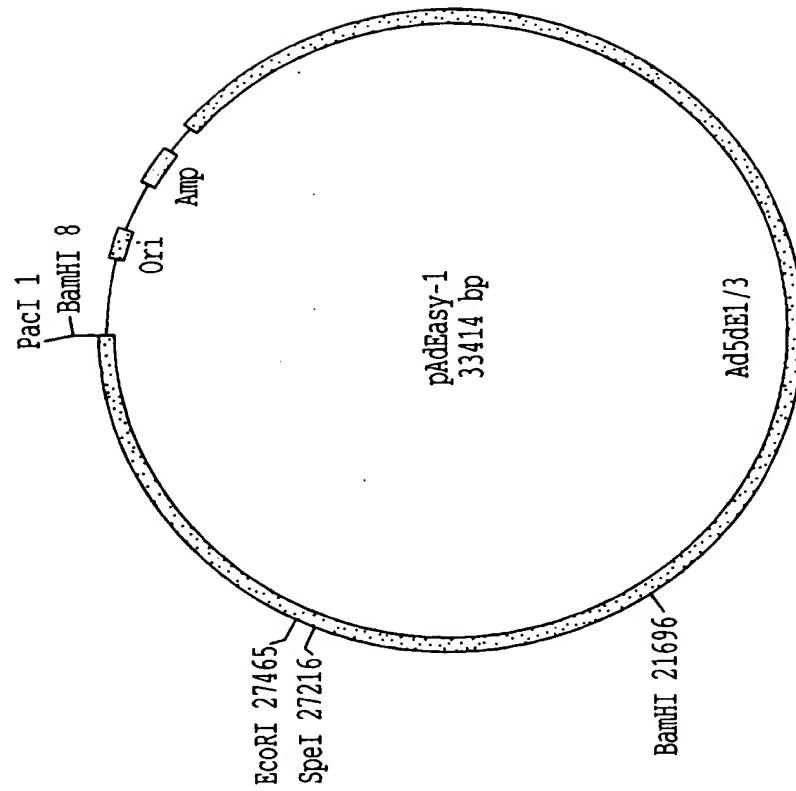


FIG. 35A

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FIG. 36A

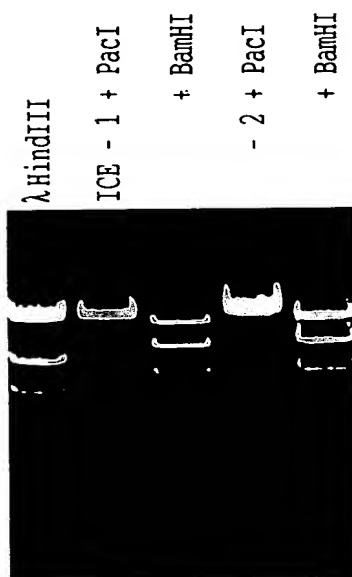


FIG. 36B

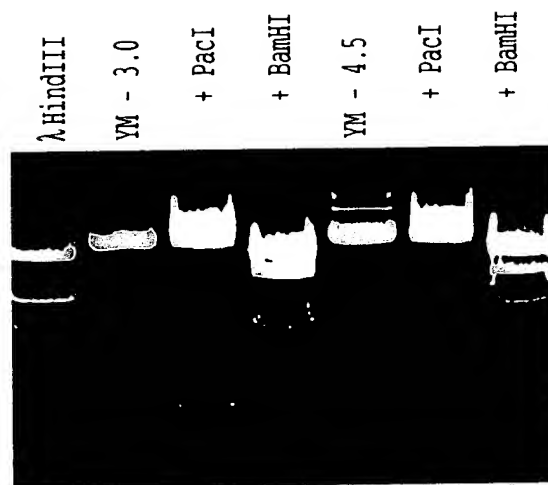
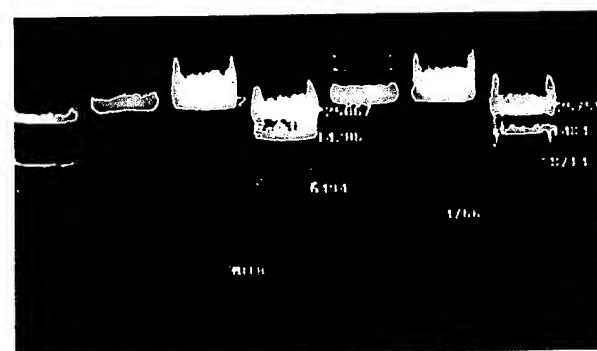


FIG. 36C



006260" 37424960

# 37/52 Generation of Ad-CMV-E-ICE

## Construction of pshuttle-CMV-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst and Ad-CMV-E-ICE

1. Cut pshuttle-CMV with EcoRV+Not I  
1μg/2μl (pshuttle-CMV) + 1μl Buffer H  
+ 1μl EcoRV + 1μl NotI + H<sub>2</sub>O 5μl  
37°C, 2hr.

2. Cut pSH1/S-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst with  
Not I + EcoRI (get E-F<sub>v</sub>1-F<sub>vis</sub>1-ICE st)

3μg/4μl + 3μl Buffer H. + 3μl EcoRI  
+H<sub>2</sub>O 21μl, 37°C, 1hr

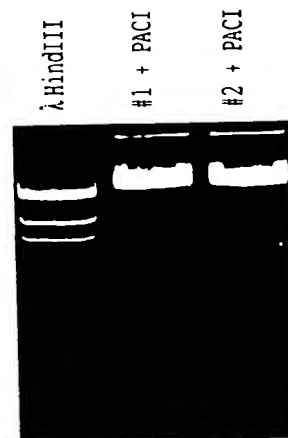


Figure 37A

### Blunt

+ 10μM dNTP 18μl (10μM/each) +3μl Buffer B  
+ 3 μl H<sub>2</sub>O + 7μl, T4 DNA polymerase, 25°C, 30 min  
+EDTA→ 10 min, 75°C, 10 min  
phenol: chloroform extraction (twice)  
ethanol precipitation → 24μl H<sub>2</sub>O

### Then

+ 3μl Buffer H + 3μl Not I  
37°C, 2hr

3. Run Gel. Cut bands, pool together  
GeneClean, Elute in 40μl H<sub>2</sub>O

4. Ligation  
Elutant 16μl+2μl 10X ligation Buffer  
+2μl T4 Ligase, 25°C, overnight

5. Transformation
6. Miniprep + Restriction Enzyme Cechking
7. Cut pshuttle-CMV-E-F<sub>v</sub>1-F<sub>vis</sub>1-ICEst with PmeI (1/5 of a miniprep), Cotransform  
E. coli BJ5183 with 100ng PAd Easy-1. Miniprep+pac I checking (Fig. 37 B)
8. Chose #2, retransform XL-I Blue, Miniprep, +pac I; Midiprep, +pac I checking  
(Fig. 37 A)

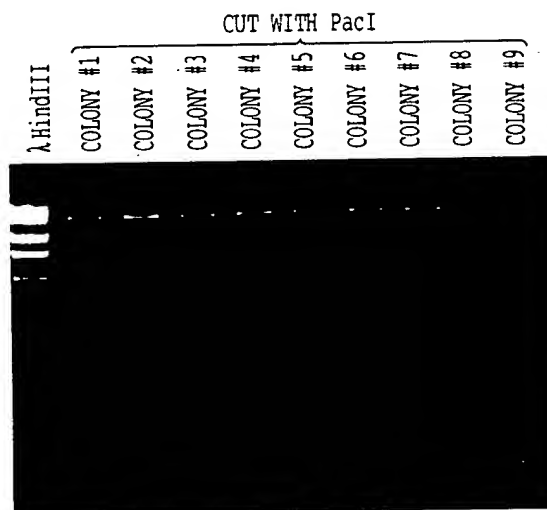


Figure 37B



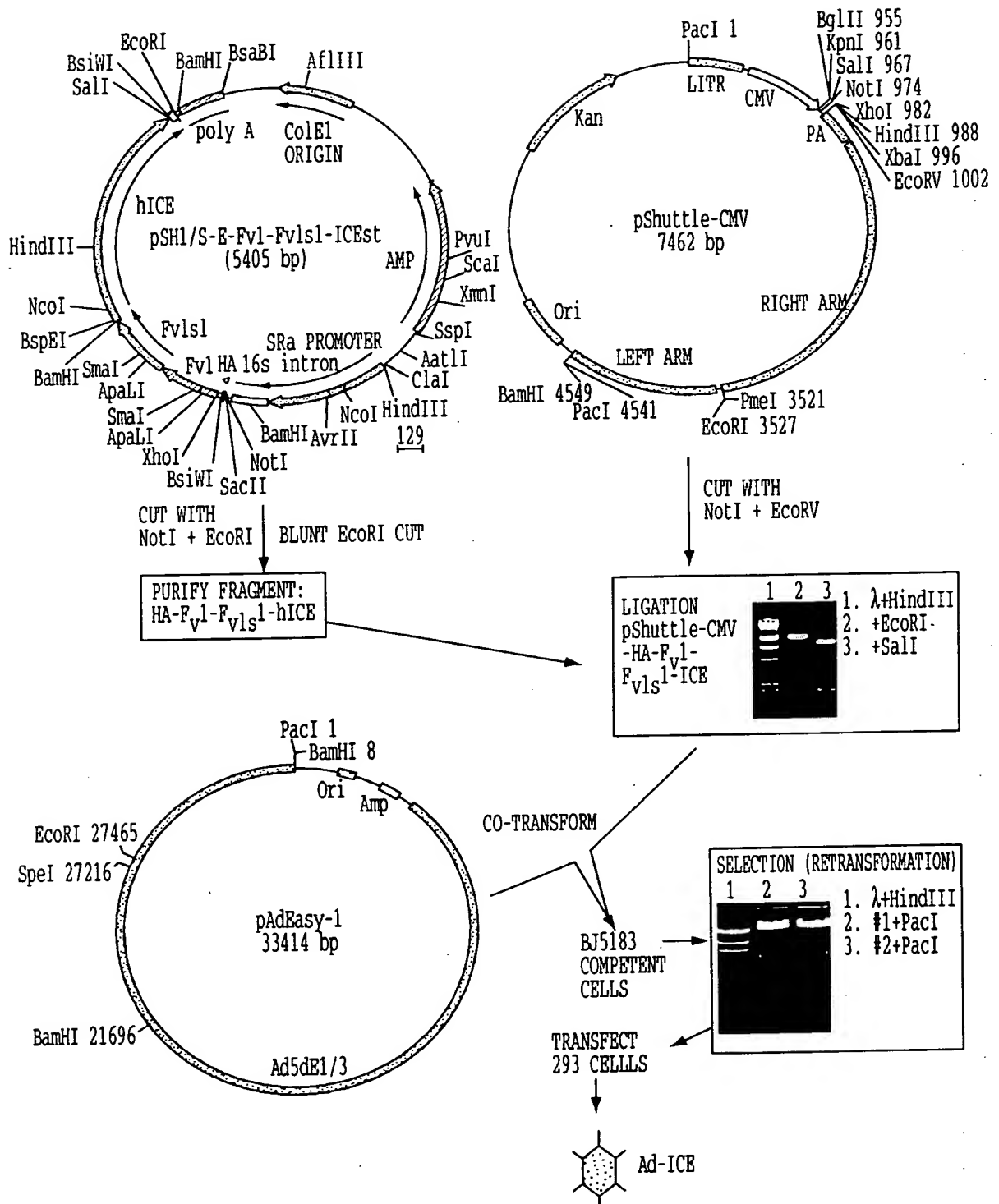


FIG. 38

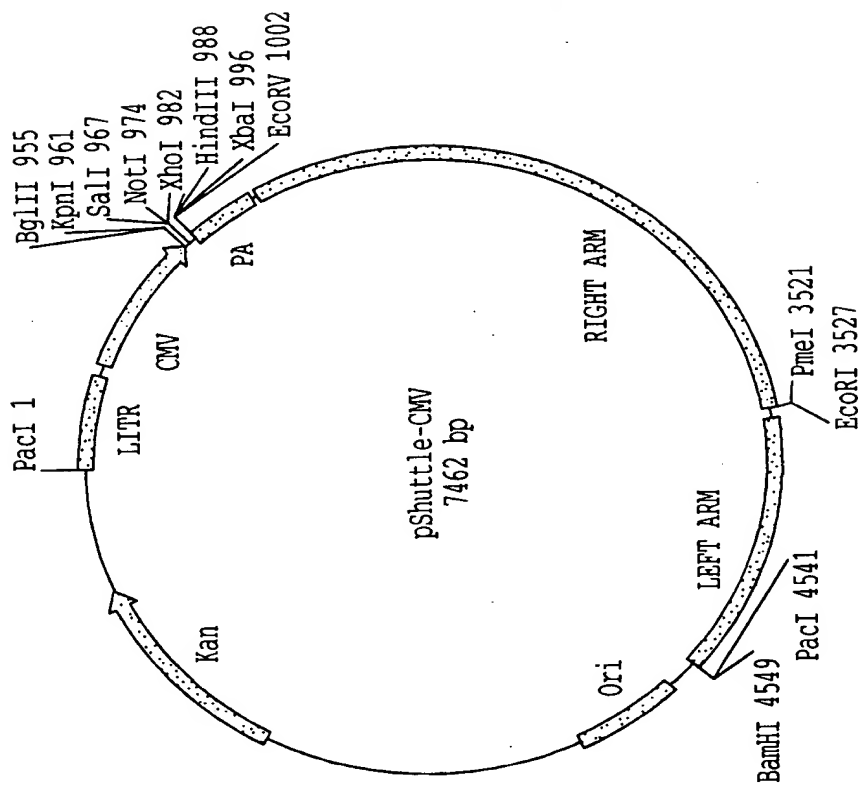


FIG. 39A

λ HindIII  
pshuttle - CMV  
+ EcoRI  
+ BamHI  
+ EcoRI + BamHI  
pshuttle  
+ EcoRI  
+ BamHI  
+ EcoRI + BamHI

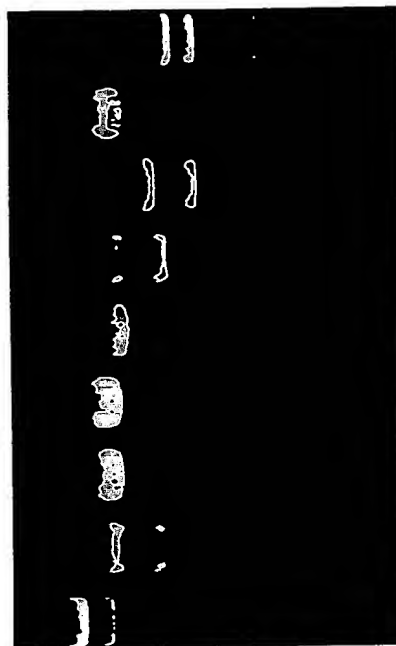


FIG. 39B

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Luciferase Assay To Determine the Effect of Different Plasmids

1. Cells were plated in 6 well-plate,  $1 \times 10^5$ / well in 3 ml media (RPMI 1640 for JD-2 $\alpha$  cells, DMEM for 293 and 293-Z4 cells) with 5% FBS, and incubated for 24 hours.
2. Cells were transfected with 2  $\mu$ g DNA each (pGL2, pTrack-ICE, pTrack-YAMA, pShuttle-ICE) and FuGene(1  $\mu$ g DNA/2  $\mu$ l) and incubated overnight.
3. AP1903, or FK 1012, or AP20187 was added at a final concentration of 50 nM and cultures were incubated 24 hours.
4. Cells were lysed and checked for luciferase activity on a luminometer.

8 JAN 99 AM 10:58

V. 1.1

MEAS. TIMES (S) : 15.0

SAMPLE		RLU	% C.V.
	1 1	294	
	1 2	291	
MEAN		292	0.8 Control
	2 1	2681958	
	2 2	2769999	
MEAN		2725979	2.3 Control + pGL <sub>2</sub>
	3 1	208762	
	3 2	223711	
MEAN		216237	4.9 pGL <sub>2</sub> + ICE
	4 1	22084	
	4 2	22859	
MEAN		22471	2.4 pGL <sub>2</sub> + ICE + AP1903

Figure 40

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Effect of Ad-GFP-Ice and YM on Prostate Cancer and BPH Smooth muscle cells  
 JD-2 $\alpha$ , T-C<sub>2</sub>, T-C<sub>2</sub>G, LNCaP-Adeno-YM or Adeno-ICE

1. Plating cells: 24 well-plate  
 JD-2a -- 20000/well in 1ml RPMI 1640 Media (5%FBS)  
 T-C<sub>2</sub> -- 10000/well in 1 ml DMEM Media (+5% FBS. 5%Nuserum, Insulin DHT)  
 T-C<sub>2</sub>G -- 10000/well in 1 ml DMEM Media (+5% FBS. 5%Nuserum, Insulin DHT)  
 LNCaP-30000/well in 1ml RPMI 1640 Media (5%FBS)  
  
 incubate JD 24 hrs                      cell number doubling - 40000/well  
 T-C<sub>2</sub>, FC<sub>2</sub>G-48 hrs.                      - 40000/well  
 LNCaP-72 hrs                              - 60000/well
2. Infection:  

For JD: ICE 1x10 <sup>7</sup> iu/ml	MOI	1	2	4	8	16	
	Add	4	8	16	32	65	$\mu$ l
YM 2x10 <sup>7</sup> iu/ml	MOI	2.5	5	10	20	40	
		5	10	20	40	80	$\mu$ l
For T-C <sub>2</sub> ICE 5x10 <sup>7</sup> iu/ml	MOI	2.5	5	10	20	40	
+ T-C <sub>2</sub> G	add	2	4	8	16	32	$\mu$ l
YM 5x10 <sup>7</sup> iu/ml	MOI	2.5	5	10	20	40	
	Add	2	4	8	16	32	$\mu$ l
YM ICE 5x10 <sup>7</sup> iu/ ml	MOI	2.5	5	10	20	40	
For LN Cap		3	6	12	24	48	$\mu$ l

After 4 hrs. Add AP 1903  $\rightarrow$  50nM. Incubate 24 hrs.
3. Fix with 1% Glutaraldehyde - PBS, 15';  
 Stain 0.5% crystal violet, 20'  
 Wash with H<sub>2</sub>O, 30';  
 Resolve with Sortson's Solution 200  $\mu$ l/well, 5';  
 Transfer 100  $\mu$ l to each well of 96-well plate, Read OD at 570 nM.

Figure 41

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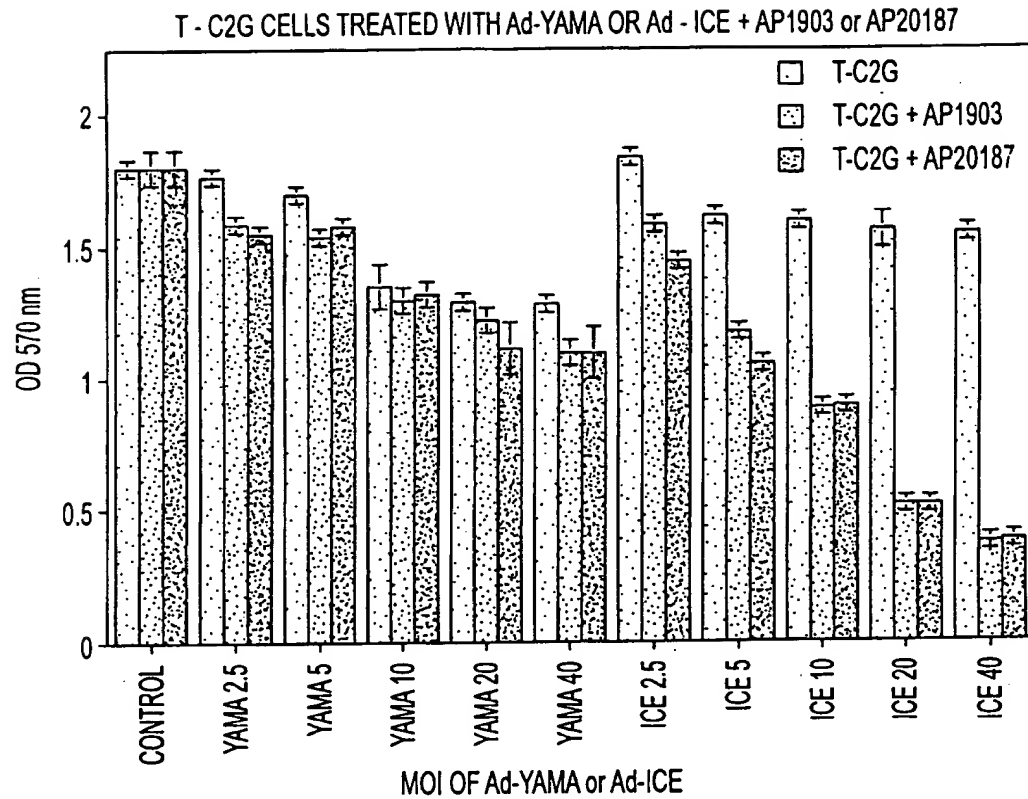


FIG. 42

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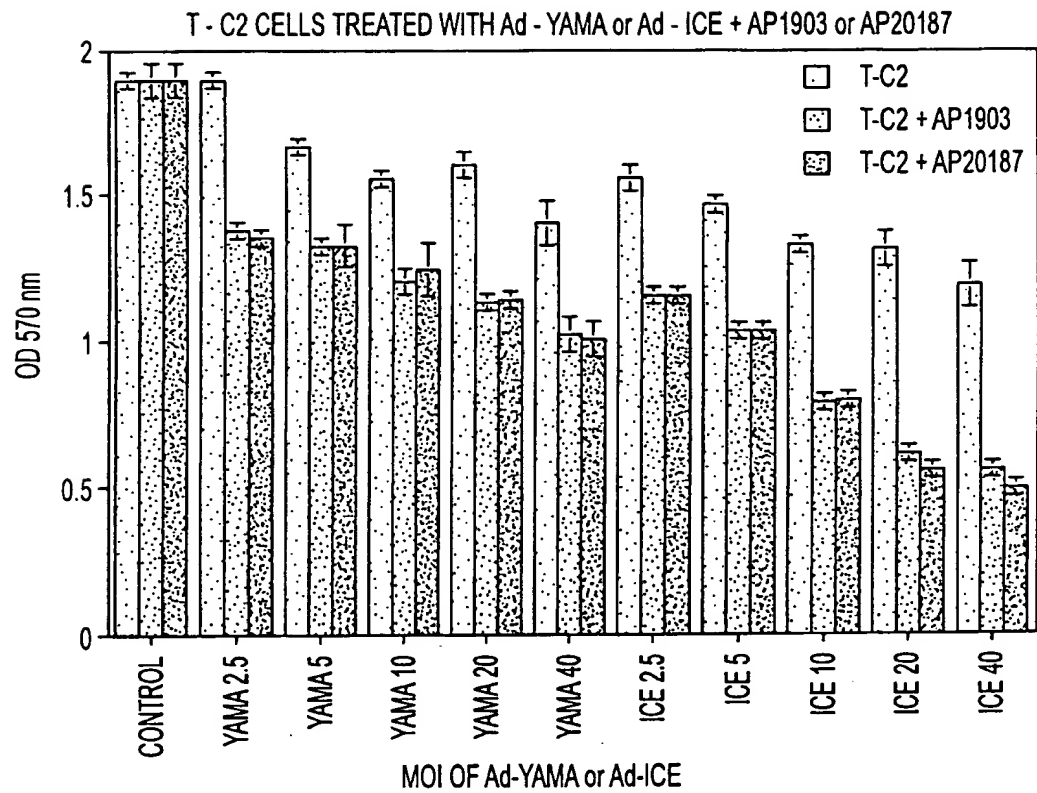


FIG. 43

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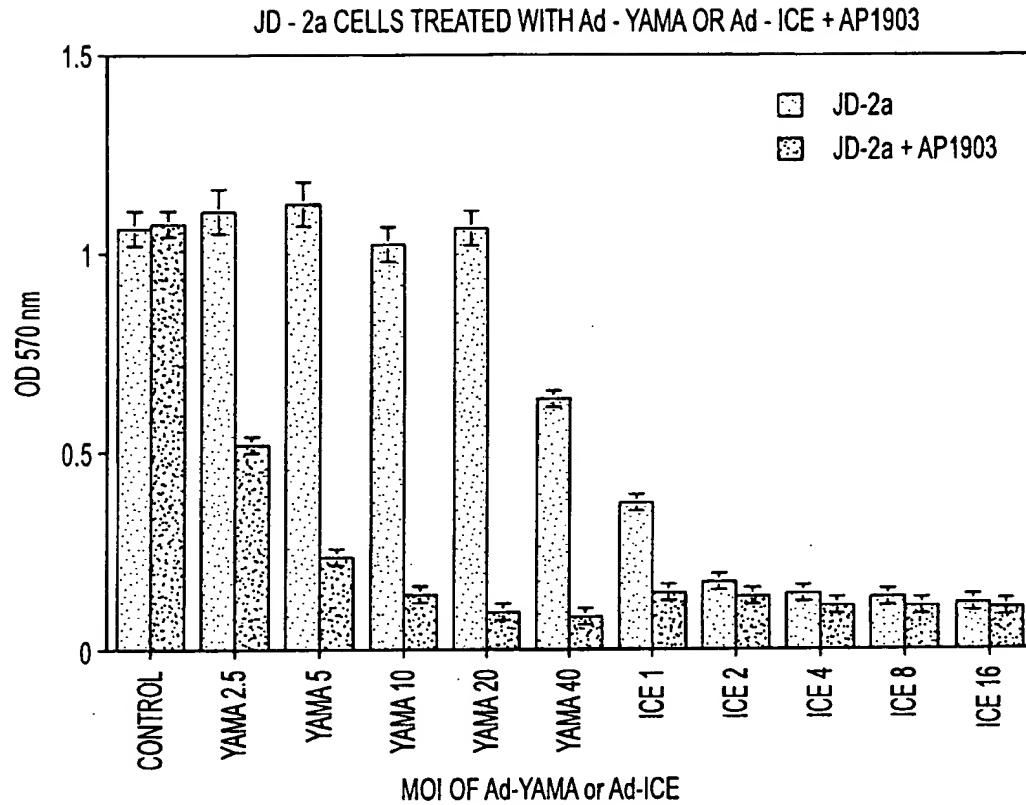


FIG. 44

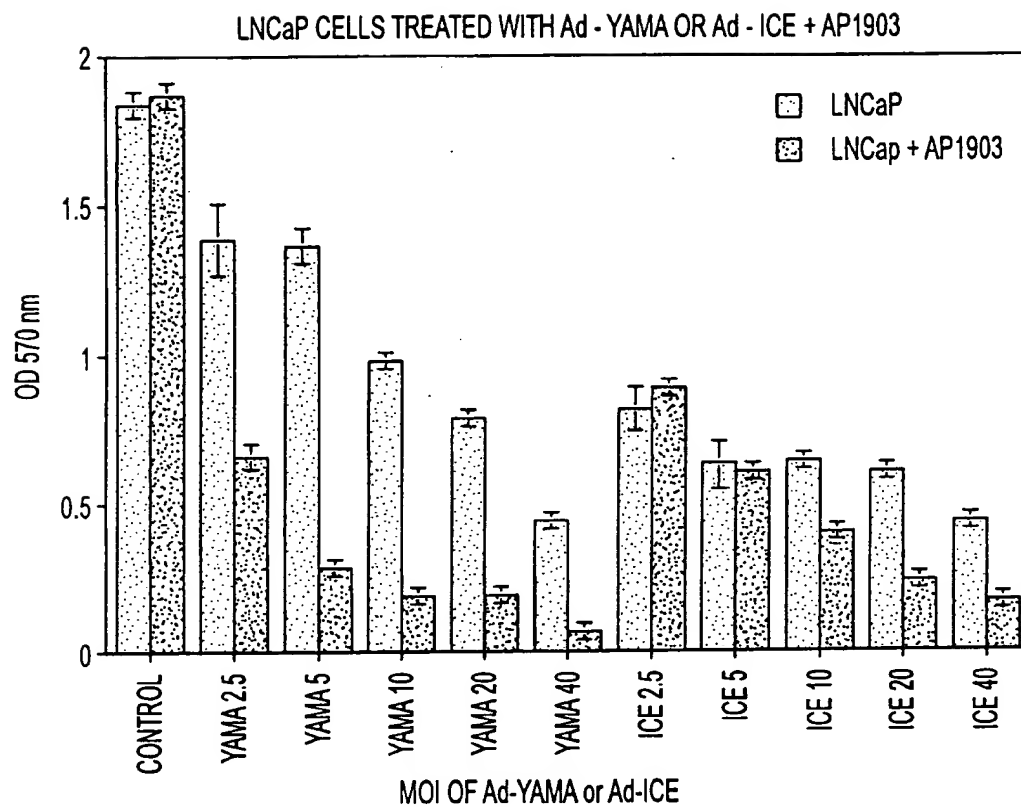


FIG. 45



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Western Blot 1103-110498

293 wells; infected with Ad-YM or Ad-ICE  
9% Resolving gel; transfer -- 400mA, 2hrs

1:1000  $\alpha$ -HA11 -- 1<sup>st</sup> Ab.

- |     |        |                 |
|-----|--------|-----------------|
| 1.  | Ad-YM3 |                 |
| 2.  | " "    | +1903(100nM)    |
| 3.  | " "    | +2D-DCB (100nM) |
| 4.  | " "    | +Both           |
| 5.  | Ad-ICE |                 |
| 6.  | " "    | +1903           |
| 7.  | " "    | +2-D-DCB        |
| 8.  | " "    | + Both          |
| 9.  | Ad-YM4 |                 |
| 10. | " "    | +1903           |
| 11. | " "    | +2-D-DCB        |
| 12. | " "    | +Both           |

EXPRESSION AND ACTIVATION OF ICE AND YM

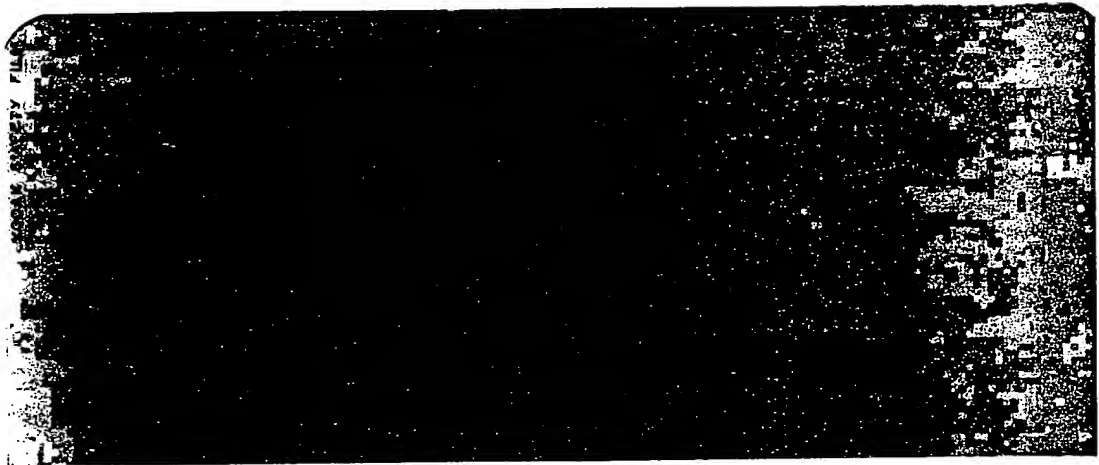


Figure 46

006260" BT74960

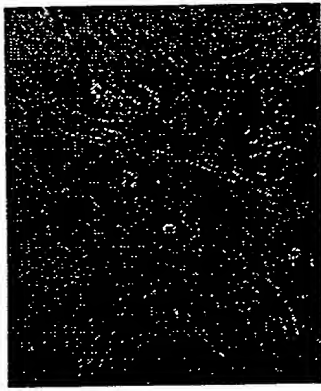


FIG. 47A



FIG. 47B

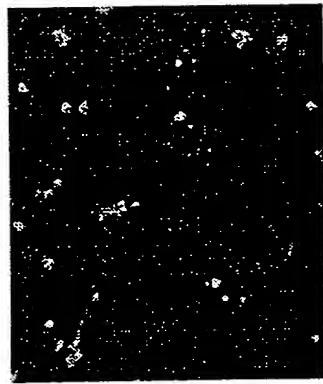


FIG. 47C

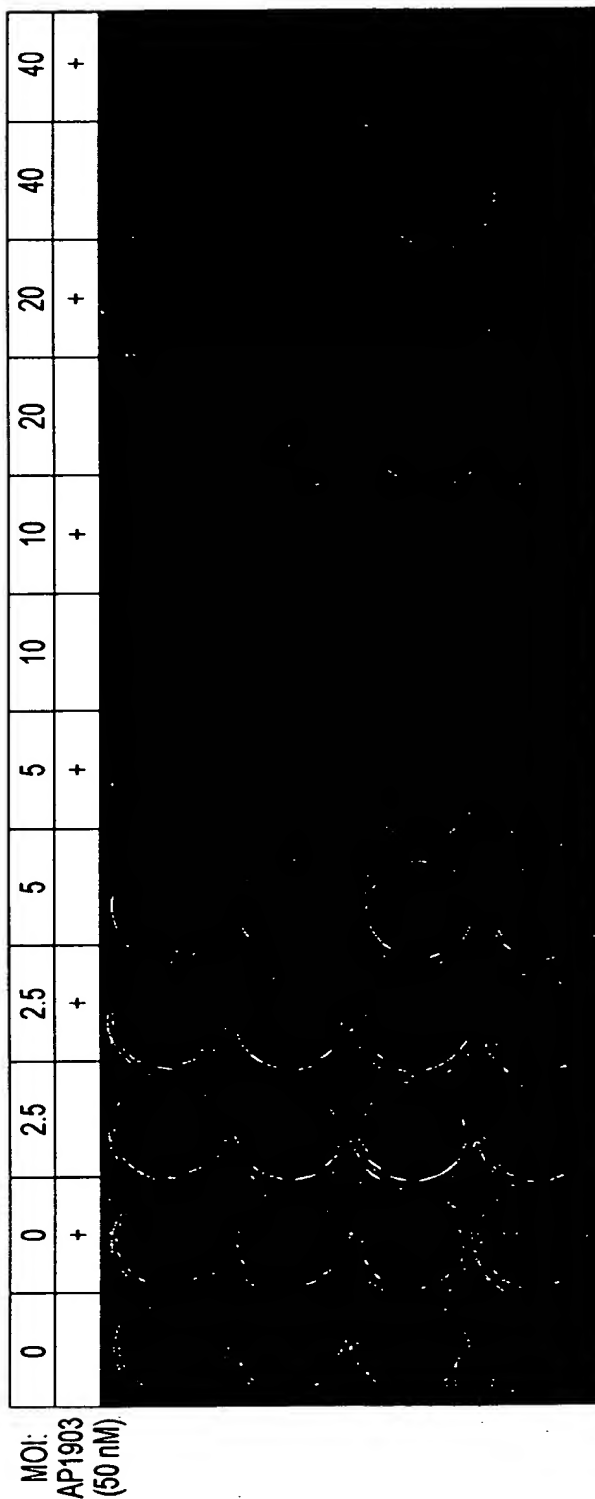


FIG. 48

006260" 8FH24960

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09/647418

0	0	1	+	2	2	4	4	8	8	16	16
	+		+		+		+		+		+

MOI:  
AP1903  
(50 nM)

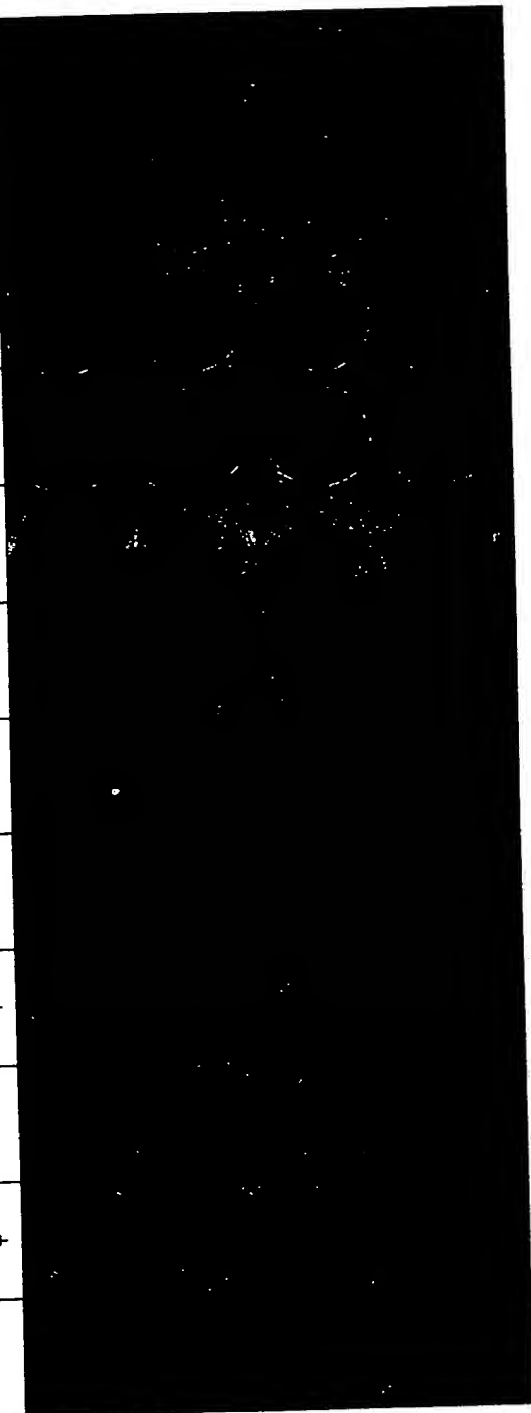
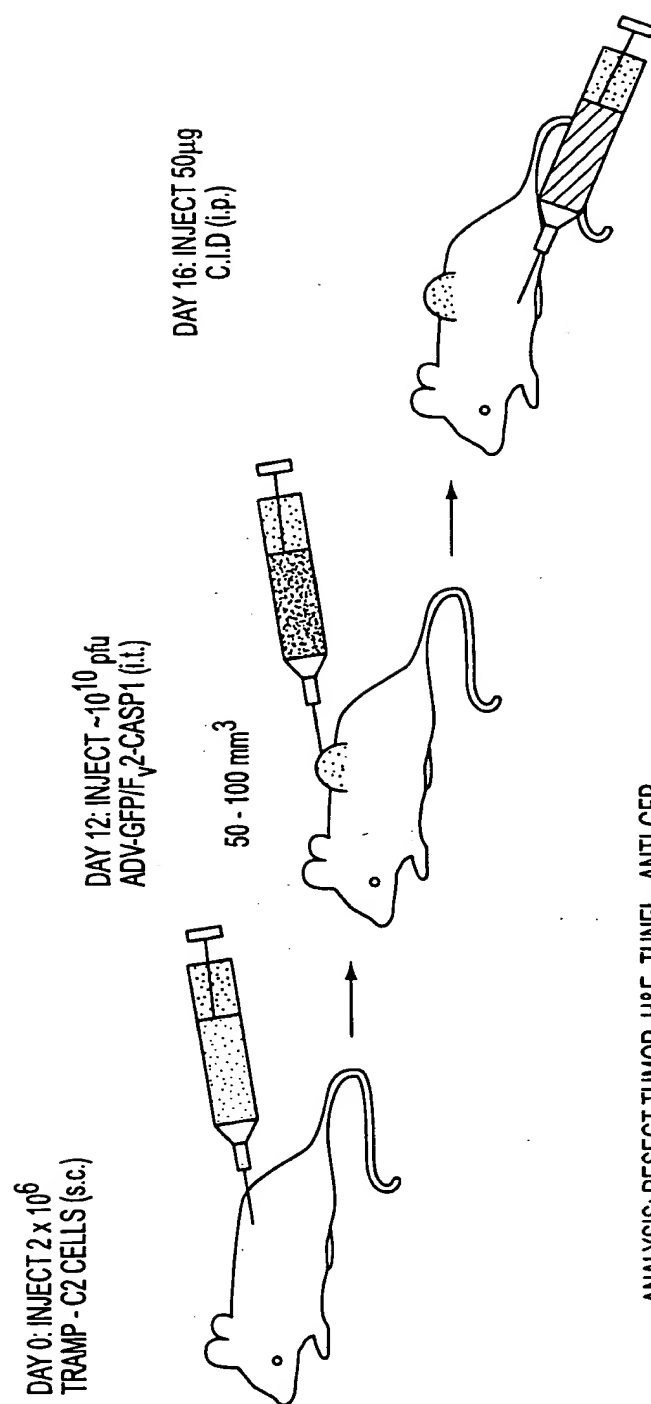


FIG. 49

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ANALYSIS: RESECT TUMOR, H&amp;E, TUNEL, ANTI-GFP

FIG. 50

006260" BT-47950

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20X

no ICE  
+ CID

FIG. 51A

20X

ICE  
no CID

FIG. 51B

10X

ICE  
no CID

FIG. 51C

40X

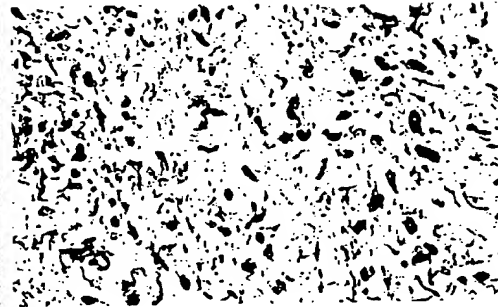
ICE  
no CID

FIG. 51D

006260" 87424960

20X

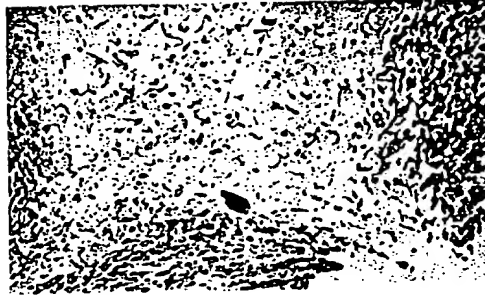
ICE  
+ CID

FIG. 52A

40X

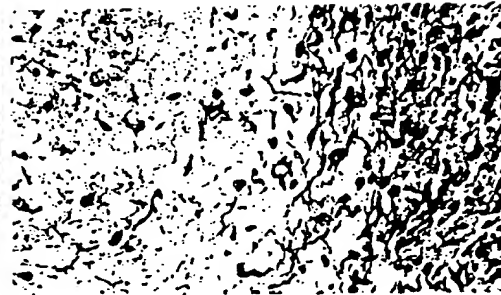
ICE  
+ CID

FIG. 52B

20X

ICE  
+ CID

FIG. 52C

40X

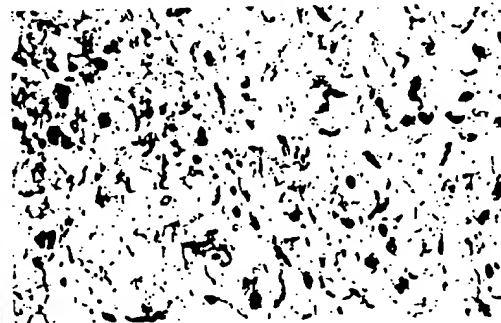
ICE  
+ CID

FIG. 52D

005250" 87424960

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